VOLUME 26 NUMBER 2

PLANT PHYSIOLOGY

APRIL, 1951

CONTENTS

Stomatal Movement and Photosynthesis in Pelargonium. I. Effects of Light and Carbon Dioxide. G. W. Scarth and Miriam. Scarth, Macdonald College, Quebec, Canada 159

Photosynthesis and Metabolism of Organic Acids in Higher Plants. R. E. Brown and R. H. Burris, University of Wisconsin, Madison, Wisconsin 175

The Effects of Some Special Treatments in the Degreening of Florida Oranges as Measured by Respiration Rate. B. E. Reese and H. V. Miller, University of Pittsburgh, Pittsburgh, Pennsylvania 195

Comparisons of Growth by Tobacco and Sunflower Tissue on Synthetic Media Containing Various Sources of Organic Nitrogen. R. M. Fraps, A. J. Riker, and S. L. Dye, University of Wisconsin, Madison, Wisconsin 209

Respiration Studies on Chlorella. II. Influence of Various Organic Acids on Gas Exchange. D. M. Eby, Cornell University, Ithaca, New York 227

Effect of Various Factors on the Utilization of Nitrogen and Iron by Spirodela polyrhiza (L.) Schleid. E. H. Eriksen and D. E. Seaton, University of Massachusetts, Amherst, Massachusetts 239

Identification of Ethylene as a Volatile Product of the Fungus Fusarium diplosporum. R. E. Young, H. K. Pratt, and J. B. Biale, University of California, Division of Subtropical Horticulture, Los Angeles, California, and Division of Truck Crops, Davis, California 262

Studies on the Physiology of Light Action. IV. Light Enhancement of Auxin-Induced Growth in Green Peas. A. W. Galston and R. B. Baker, California Institute of Technology, Pasadena, California .. 281

(*Continued on Cover Page Four*)

PUBLISHED QUARTERLY

BY

THE AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS

AT 111 E. CHESTNUT ST., LANCASTER, PA.

Entered as second-class matter March 25, 1926, at the post office at Lancaster, Pa., under the Act of August 24, 1912.

Accepted for mailing at the special rate of postage provided for in paragraph [illegible], section [illegible], Authorized April 6, 1926.

PLANT PHYSIOLOGY

A QUARTERLY JOURNAL
DEVOTED TO ALL PHASES OF PLANT PHYSIOLOGY

Established 1926

WALTER F. LOEHWING, *Editor-in-chief*, 1954
State University of Iowa

ASSOCIATE EDITORS

C. B. WADLEIGH, 1955
U. S. Regional Salinity Lab
Riverside, California

M. W. PARKER, 1954
U.S.D.A. Plant Industry Station
Beltsville, Maryland

J. B. HALE, 1953
University of California
Los Angeles, California

IRENE H. STUCKEY, 1952
Rhode Island Agricultural
Experiment Station

H. B. VICKERY, 1956
Connecticut Agricultural
Experiment Station

F. P. CULLINAN, 1955
U.S.D.A. Plant Industry Station
Beltsville, Maryland

C. S. FRENCH, 1953
Carnegie Institution
Stanford, California

F. C. GUSTAFSON, 1952
University of Michigan
Ann Arbor, Michigan

ROBERT M. MUIR, *Secretary of the Editorial Board*, 1956
State University of Iowa

PAUL J. KRAMER, 1953
Duke University

HERA J. KRAUS, 1952
Oregon State College

WALTER E. LOOMIS, 1951
Iowa State College

The pages of this journal are open to papers of merit dealing with any phase of Plant Physiology. Membership in the Society is not required of contributors, but non-members are required to pay a fee of $20.00 for each paper printed.

Manuscripts must be typewritten, and must have the approval of the Editorial Board. Papers must conform to the limitations established by the Society at the St. Louis meeting, 1926.

Proofs should be returned with necessary corrections, and reprints ordered when the proof is returned. Reprints can be furnished with or without covers at cost of production.

Correspondence with reference to editorial matters should be addressed to the Editor, Dr. Walter F. Loehwing, Department of Botany, State University of Iowa, Iowa City, Iowa.

Subscription price, $10.00 a year. Single copies, $3.00 post free. Members of the American Society of Plant Physiologists receive the journal in connection with dues paid to the Society.

Missing numbers will be supplied if lost in the mail, provided that written notice is received within one month of the date of issue; otherwise they will be furnished at the single copy rate, as long as they are available.

Correspondence concerning membership and its privileges, dues, change-of-address, etc., should be addressed to the Executive Secretary-Treasurer, Dr. J. Fredrik Stanffer, Miami University, Oxford, Ohio.

Business correspondence regarding subscriptions, back numbers, change-of-address of subscribers, etc., may be addressed to PLANT PHYSIOLOGY, 111 E. Chestnut St., Lancaster, Pa., or to the Executive Secretary-Treasurer at the address above.

All matters pertaining to nominations, elections, programs, and meetings of the society should be addressed to the Secretary, Dr. T. C. Broyer, University of California, Berkeley, California.

GEORGE WILLIAM SCARTH

IN CELEBRATION OF

HIS SEVENTIETH BIRTHDAY

MAY 29, 1951

On May 29th of this year George William Scarth will celebrate his 70th birthday. Plant physiologists the world over will congratulate him and wish him many years of leisure. Born in the Orkneys, he spent most of his school days at Kirkwall. Physically and mentally, like many other Orkney men, he is a Norseman rather than a Scot. His undergraduate years were passed at Edinburgh, along with Jack Bews (author of "The World's Grasses"), the late John Tait (subsequently for many years Professor of Physiology in the same building at McGill), and Principal R. C. Wallace of Queen's University, Kingston, Ontario. While at Edinburgh Scarth was for some time an assistant of Bayley Balfour. His first paper was on the grassland of Orkney, and he was subsequently to play an important part on pasture committees in Quebec. One scarcely thinks of him now as an ecologist but two further papers (1922) were on ecological topics.

He came to McGill some 30 years ago to join Professor F. E. Lloyd and here he had to plan and teach one of the earliest courses in cellular physiology, publishing with Lloyd "An Elementary Course in General Physiology" in 1930. At McGill he became interested in colloids (anyone working with Lloyd was bound to) and his papers reflect that interest, including as they do such titles as "Colloidal changes associated with protoplasmic contraction," "The elasticity of gelatin in relation to pH and swelling," and "The mechanism of accumulation of dyes by living cells." During the next few years he turned his attention to the cell-wall and to the study of the penetration of water into floated logs. The present writer is happy to have collaborated as a junior author in three of the papers which resulted from this work. It was at this time, too, that Scarth came to know and love stomata—a love that remains as strong as ever (see p. 207 in this number).

It was not inappropriate for one working in Montreal to become interested in frost resistance! With the assistance of such graduate students as Jacob Levitt (himself the author of "Frost Killing and Hardiness of Plants," 1944) and David Siminovitch, several papers appeared on this topic in the years between 1936 and 1944, culminating in a review of the subject—"Cell physiological studies of frost resistance."

From the beginning his work at McGill included a considerable and at times a heavy program of teaching. His elementary lectures were perhaps well above the heads of all but a very few in his classes, but at the advanced and graduate levels he inspired a generation of students. Lloyd retired in 1934 and Scarth became Macdonald Professor of Botany and Head of the Department.

Scarth is one of the early members of the American Society of Plant Physiologists; he was vice-president in 1937–38, and was for many years a member of the Editorial Board of PLANT PHYSIOLOGY. In 1944 he was made

the 22nd Charles Reid Barnes Life Member of the Society—an honor which he values highly. At that time McGill had (in Lloyd and Scarth) two Barnes Life Members—a distinction shared by no other University. He was awarded a D.Sc. by McGill in 1933. Elected to the Royal Society of Canada in 1928, he became President of Section V (Biological Sciences) in 1935–36, his presidential address being entitled "The Yearly Cycle in the Physiology of Trees."

Scarth retired from McGill in 1946 and was subsequently made a Professor Emeritus of the University. He has lived for many years at nearby Ste. Anne de Bellevue where Macdonald College—a part of McGill—houses the Faculty of Agriculture, the School for Teachers, and the School of Household Science. For many years he has lectured there, as a labor of love, in plant physiology, and he continues to work, with a graduate student or two and his beloved stomata. In recent years he has been improving apparatus for the measurement of photosynthesis, respiration, and transpiration, using the infra-red total absorption method. With this he is probing more deeply into the mechanism of stomatal movement (p. 207). This infra-red apparatus is elaborate and rather costly but Scarth was never one to depend upon complicated gear. Many of his most beautiful experiments were made with imagination and the simplest of materials!

Scarth married Margaret Ramsay in 1920 and their home has been a home-away-from-home for many a student. They have three children, all of them graduates of McGill, and the youngest should add a degree in Medicine to his Arts degree this spring. It would be an odd McGill without a Scarth as teacher or as student, and it is to be hoped that Scarth himself will be able, for many years to come, to continue his present happy association with Macdonald. The present writer looks forward to the time when he can pen a tribute on the 80th birthday of one whom he has known as mentor and friend for more than 25 years.

R. Darnley Gibbs

PLANT PHYSIOLOGY

VOLUME 26 **APRIL, 1951** **NUMBER 2**

STOMATAL MOVEMENT AND PHOTOSYNTHESIS IN PELARGONIUM. I. EFFECTS OF LIGHT AND CARBON DIOXIDE[1]

GEORGE W. SCARTH AND MICHAEL SHAW

(WITH SEVEN FIGURES)

Received May 9, 1950

The old hypothesis that light induces stomatal opening through its photosynthetic activity has been variously tested in a number of recent investigations. The results are strangely contradictory. First, reference may be made to tests based on the relative efficiency of different regions of the spectrum in promoting stomatal opening, though these tests are not very recent. The only systematic studies in which strictly quantitative methods have been used to equalize light intensity are those of PAETZ (**11**), SIERP (**15**), and HARMS (**6**). Paetz found that the order of activity was red light > blue > green, whereas Sierp and Harms found that red light was regularly the least active and that in most species green light was as active as blue.

It has long been known that a reduction in the normal CO_2 content of the air tends to induce stomatal opening and an increase in CO_2, up to a point, above normal has the opposite effect. It has, however, only recently been demonstrated (HEATH, **7**, **9**) that the sensitivity of stomata to small changes in CO_2 concentration below normal is such that stomatal response to variations in light intensity could conceivably be due primarily to the effect of the latter on CO_2 concentration within the leaf.

The only investigation in which measurement of photosynthesis rate has been combined with estimation of stomatal aperture is that of WILSON (**18**). Approaching the problem from various angles, he obtained results which seem quite opposed to the photosynthetic theory. Both in the behavior of plants in the open and under certain artificial conditions, stomatal opening and photosynthesis often showed no correlation, while complete absence of chlorophyll from leaves, including guard cells, did not entirely prevent stomatal response to light. On the other hand, regarding the long unsettled question of whether guard cells are capable of photosynthesis, the most

[1] Contribution from the Faculty of Agriculture, McGill University, Macdonald College, Quebec, Canada. Journal Series No. 284.

recent work (ALVIM, **1**; FREELAND, **2**) indicates that the answer is probably affirmative.

The present paper reports a preliminary reconnaissance of the whole field by means of a new technique, a technique which allows the time courses of photosynthesis and stomatal movement to be followed more or less continuously in the same leaf.

General methods

The plant material used in these experiments was single attached leaves of *Pelargonium zonale* var. Barney, all from plants of the same clone. The

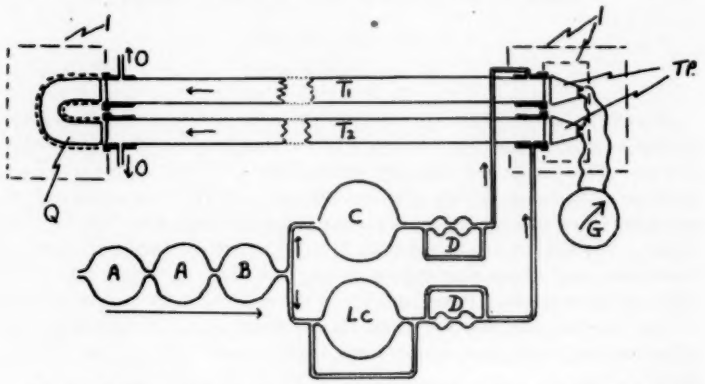


FIG. 1. Diagram of the apparatus used for measuring CO_2 by infrared absorption, and part of the air stream system. A. Humidifying chambers into which air is pumped. B. Spray trap. L.C. Leaf chamber. C. Compensating chamber for equalizing the time lag in the two branches of the air stream. D. Drying units (containing barium chloride and magnesium perchlorate) and by-pass tubes. T_1 and T_2. Internally polished steel absorption tubes which have mica windows at each end. O. Outlets through which air is pulled (via two flowmeters and two manometers) by a constant-head suction pump. Q. Quartz tube heater surrounded by resistance coils. I. Insulating boxes. TP. Thermopiles. G. Galvanometer.

experimental leaf was usually enclosed (with porometer cup attached and thermocouple junction appressed to it) in a circular glass leaf chamber, which was then sealed except for the inlet and outlet tubes through which air was passed.

The apparatus used for measuring photosynthesis was essentially the same as that described in a previous paper (**13**). There was, however, an important improvement in the arrangement of its parts. Figure 1 shows the pair of infrared absorption tubes (T_1 and T_2) placed parallel, with a U-shaped heater (Q) at one end and two thermopiles (Tp) at the other. The juxtaposition of the thermopiles, which in the original design were far apart, has greatly reduced disturbances due to extraneous temperature

effects. The heater, as before, was an electrically heated quartz tube filled with CO_2. Though it has been found that the CO_2 content of the heater does little in providing wave lengths of radiation that are absorbed by the gas, yet at a temperature of 900 to 1000° C the peak of emission by the whole heater is about the same as that of absorption by CO_2, so that it makes a fairly efficient source and one with very constant intensity of radiation. In measuring photosynthesis one absorption tube is filled with normal air, which has been dried, and the other with air which has passed over the experimental leaf before being dried. The differential current set up by the paired thermopiles with their E.M.F.'s opposed is then governed by the difference in CO_2 concentration in the respective tubes, and a calibration curve enables the effect on the galvanometer to be translated into CO_2 concentration. In measuring transpiration the difference in concentration of water vapor is determined in the same way, using the appropriate calibration curve.

Normal air in the sense here used means "country" air pumped in from outside the building. It is bubbled through saturated salt solutions to obtain the desired water vapor pressure—which is easily measured on the apparatus. A continuous stream of this air is passed at a constant measured rate of flow through the apparatus, the pressure in the two absorption tubes being kept the same and that in the leaf chamber equal to atmospheric pressure by means described in the paper cited above. The present diagram shows part of the air flow system. It is easily possible to measure accurately less than one part of CO_2 per million of air at normal atmospheric concentration of CO_2, if the apparatus zero is checked at the time of measurement.

Stomatal aperture (or, more precisely 1/R, R being the resistance of the leaf to air flow) was measured porometrically on the same leaf in intervals between the measurements of photosynthesis. The porometer technique has also been described elsewhere along with diagrams of its apparatus and of the leaf chamber (SHAW, **14**). An important feature is that, except for the brief intervals required to make measurements, the porometer cup is continuously swept with air led in through a narrow side arm of the main stream. The air is renewed at the same rate in the cup as in the surrounding chamber, with the result that the behavior of the stomata inside and outside the cup is the same. This is often not the case when the air in the cup is allowed to stagnate, or only a slow stream is passed through the leaf.

The rate of air flow used most commonly was 50 liters per hour. Rates refer to volumes measured at room temperature and normal pressure. Such a rate gives a wind velocity of 46 cm. per minute across the mean diameter of the standard leaf chamber (8 by 3 cm.). At this rate of flow removal of atmospheric CO_2 by photosynthesis never reached 20% and usually did not rise above 12%. With 12% removal, at least, no appreciable difference in stomatal opening was found in different parts of the leaf. Sometimes the whole upper and lower surfaces differed from one another to some extent,

but any large difference in this respect (which seemed to be associated with a semipathological condition of the leaf) led to discarding of the experiment. Measurements of 1/R with a wide-rimmed porometer cup on one and the same leaf of the variety of Pelargonium used were found to vary approximately as the area of the pores. In experiments pore area ($\pi/4 \times$ length $\times$ width) was determined by measurement (Lloyd's method) at one or more values of 1/R and calculated by simple proportion for others. Conversions from mean diameter to area or the reverse were made from calibration curves.

The source of light was generally a daylight fluorescent lamp. Four 40-watt tubes beneath a reflector gave an intensity of 500 fc at the level of the leaf when placed at the closest convenient working distance. For higher light intensities, Mazda lamps and occasionally sunlight were used. Temperature control under the fluorescent lamp was adequately achieved by varying the rate of flow of cold water through a screen placed between the lamp and the chamber. Under hotter sources the addition of a jet of air from an electric drier was sometimes required. Leaf temperature was measured regularly by means of thermocouples. Under the fluorescent lamp the leaf was generally 0.2 to 0.5° C cooler than the body of the chamber. Different parts of the leaf varied no more than 0.2° C.

Experiments and results

In the following experiments the relationship between photosynthesis rate and stomatal aperture has been sought by varying one factor at a time and measuring the effect on one or both of these. The experiments and results are classified according to the environmental factor that was varied. The present paper deals with the effects of varying the intensity and quality of light and the concentration of carbon dioxide.

Light

Pore area vs. mean diameter.—In estimation of the correlation that may exist between stomatal aperture and any other variable the result will differ somewhat with the pore dimension that is used. The two experiments that are first compared bear on these relationships as regards photosynthesis, and therefore on our main question.

In one experiment a leaf, which had been in darkness overnight, was suddenly exposed to a light intensity of 500 foot-candles from the fluorescent lamp. In the other experiment the leaf was exposed to the natural gradual increment of daylight. Photosynthesis and porometer measurements were made at intervals as recorded in figure 2. For ease of reference, individual leaves that appear along with other leaves in any figure or table are each identified by a particular letter, which is used also for the same leaf in other experiments but is not repeated for a different leaf.

With leaf A, which was suddenly illuminated, the time curves of photosynthesis and of the mean diameter of the stomatal pores almost coincide

on the scale used. The 1/R or pore area curve cannot be made to agree so well. The former agreement is made the more striking by the steps that occur in both curves. As in all our experiments, there was no measurable uptake of CO_2 until the stomata began to open, so that the rate of photosynthesis must have been limited by the diffusive capacity of the stomata during opening, at least in its early stages. Its rise had to keep pace with the widening of the pores, and when this paused in the middle of its course (a not uncommon occurrence) the rise in photosynthesis was also arrested.

In the case of natural day opening (B in the figure) the general form of the curve of 1/R agrees much better with that of photosynthesis than the

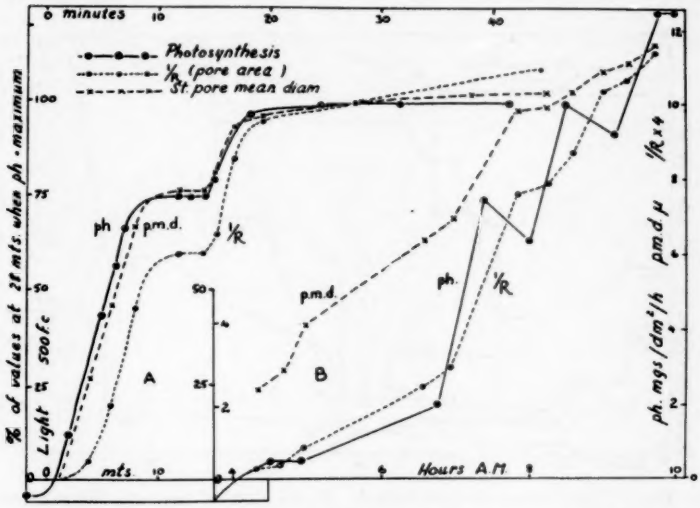


FIG. 2. Time courses of photosynthesis and stomatal opening (1/R and pore mean diameter) under: A. Light of 500 fc intensity applied at zero minutes. 100% = 5.0 mg./dm.²/hr. CO_2 uptake, and 6.9 μ pore mean diameter. B. Natural daylight. Sunrise 4:25 A.M. Hours are standard time.

mean diameter curve does. During the early stages of opening the rapid elongation of the stomatal slit causes mean diameter to increase very rapidly so that in this case it reached 2.5 μ before sunrise while photosynthesis was still low. It is evident therefore that here photosynthesis was limited by light intensity rather than CO_2 supply. After about 6:45 A.M., when direct sunlight first fell on the leaf chamber, the photosynthesis rate rose rapidly. Subsequent dips represent periods when the chamber was lightly shaded by the tops of cedar bushes. All the stomatal responses show a considerable lag. These results suggest that whereas the diffusive capacity of stomata is more nearly proportional to their mean diameter than to

their area, the effect of light intensity on stomatal aperture is probably better measured in terms of pore area.

EFFECT OF LIGHT INTENSITY.—For an indication of whether the degree of stomatal opening at equilibrium has closer correlation with light intensity or with the rate of photosynthesis, a leaf was exposed to three different light intensities until it attained equilibrium with each. This was done twice with each of two leaves, the pretreatment having been varied each time. Time curves of photosynthesis and pore area are shown in figure 3. The C_1 curves are from a leaf taken from diffuse light outside at 8:30 A.M. in midsummer. The C_2 curves are from the same leaf next day after it had been kept in darkness for 20 hours. The D_1 curves represent a leaf that was

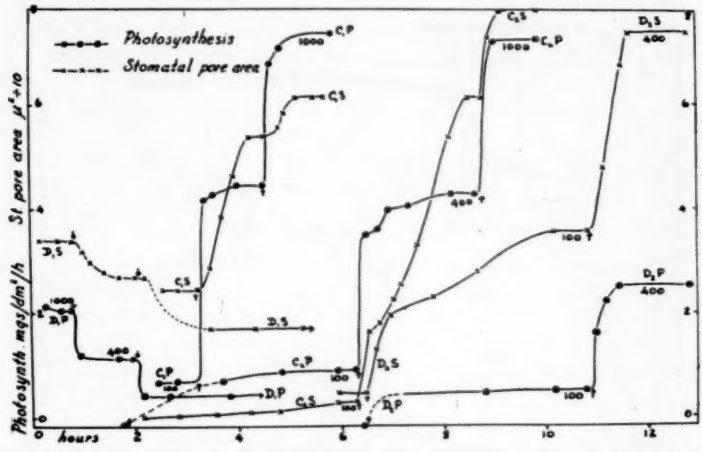


FIG. 3. Time courses of photosynthesis (P) and stomatal pore area (S) in adjustment to light intensities of 100, 400, and 1000 fc. Arrows show when the light was changed. Intensities are marked at equilibrium levels of P or S. C_1 and C_2 represent one leaf and D_1 and D_2 another leaf, each after two different pretreatments (see text). Hours are merely time intervals; all runs except D_1 started before 9 A.M.

taken from strong sunlight at 10:20 A.M. and set up under light of 1000 fc. The light intensity was decreased by stages in this case, while it was increased in the others. D_2 is the same leaf after 20 hours in darkness.

From the above data pore area at equilibrium is plotted against light intensity in part I of figure 4 and against photosynthesis rate in part II. Somewhat arbitrary smooth curves link the points of each separate experiment. One significant point to be noted regarding the curves is that three of them, though diverging widely (notably in the case of D_1) when plotted against light intensity, fall fairly well into line when plotted against the rate of photosynthesis. Another point is that the curve D_2, which stands higher than the others, and bears about the same relation to their average

on the light intensity as on photosynthesis scale, shows a considerable degree of opening existing before the light was turned on. This opening suggests that the same unknown, presumably internal, factor which was responsible for the opening in darkness, continued to operate in light and intensified its effect. Of these few results, therefore, it may be said that, as regards the action of light itself on stomata, they are at least consistent with the photosynthetic hypothesis.

EFFECT OF LIGHT QUALITY.—So as to obtain a wide spread of photosynthetic activity, green and red light were chosen for comparison, along with the white light of the daylight lamp. The green quality was produced by filtering through a chlorophyll solution, namely a layer 1 cm. thick of an extract in 95% alcohol of dried leaves of Pelargonium. The red light had passed through a glass filter which, in combination with the daylight source,

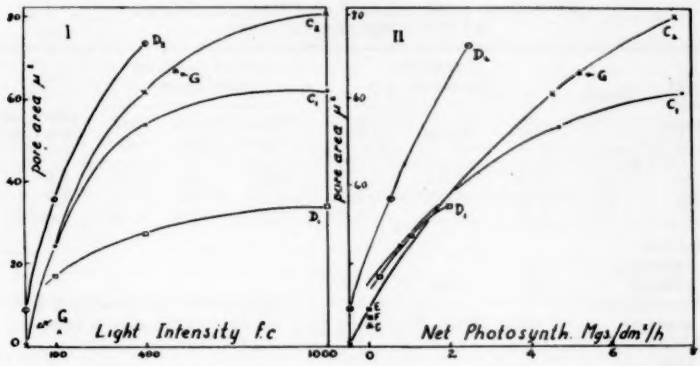


FIG. 4. Comparison of the relation of equilibrium pore area to light intensity (I) and photosynthesis rate (II). The C and D curves are from data in figure 3. Isolated points E, F, and G are for other leaves.

gave maximum transmission between 6450 and 6500 Å, and a range extending from about 5400 to 8400 Å. The green screen absorbed 70 to 75%, and the red one over 90% of the incident light.

The composition of the light from the source was obtained from General Electric Lamp Bulletin LDI; that of the red light and also the distribution of sensitivity of a Weston photocell were estimated from measurements with a spectrophotometer. Transmission by the chlorophyll solution was not measured at the time, but, as it happens, a rough estimate was sufficient in this case.

Owing to the difficulties inherent in precise measurement or equalization of the intensities of different regions of the spectrum, it was decided to equalize photosynthesis rates under the different kinds of light (which could be done easily and accurately with the apparatus), while estimating relative light intensities at leaf level by means of both thermopile and photocell.

Also, since with the fluorescent lamp and red filter only low light intensities and photosynthesis rates could be obtained, the compensation point was taken as the most convenient basis of comparison, because when the air is allowed to stagnate in the leaf chamber for a few minutes before it is passed on to the absorption tube, the light condition which produces neither uptake nor release of CO_2 can be determined with great precision. As the results show, considerable stomatal opening occurred at the compensation point, and variations of $\pm 4\%$ in 1/R could be detected. In practice the lamp was brought nearer to the leaf by small stages until net carbon dioxide exchange became zero. This was checked at intervals until the porometer reading had become constant and remained so for 30 minutes. The process was repeated for each quality of light with the same leaf.

TABLE I

EFFECT OF LIGHT QUALITY ON STOMATAL OPENING AT THE COMPENSATION POINT.

Leaf and Expt.	Temp. °C	Radiation thermopile μ v			1/R (± 0.005) at equilibrium			p.m.d. μ ± 0.1 (same for each light)
		White	Green	Red	White light	Green light	Red light	
E_1	18	3.45	6.00	....	0.10	0.10	0.10	4.5
E_2	19	3.55	6.60	3.1	0.12	0.12	0.12	4.9
F	19	3.45	6.75	3.2	0.25	0.24	0.24	5.0–4.9
Relative radiation average		100	185	90				
Photocell fc average		44	85 ($\times \frac{10}{11}$)	29 ($\times \frac{4}{3}$)	Difference in 1/R between E & F is due to difference in stomatal frequencies.			
Relative light intensity corrected		100	173	88				

Table I gives the measurements made at the compensation rate of photosynthesis in two comparisons with one leaf E and one with another leaf F. The relative values of total radiation measured with a Moll large-surface thermopile are useful mainly as a check on the photocell measurements. The latter were made with a General Electric exposure meter, type DW48, which was calibrated by comparison with the Weston photocell mentioned above. The table gives the averages of the actual readings and (in brackets) the correction used to convert these to relative light intensities. The intensity of the red light was not much lower than that of the white, owing probably to the width of the transmission band, which, though its peak is close to that of photosynthesis, includes also wave lengths that are relatively inactive. The intensity of the green light is very different, being about twice that of the red.

The main result of the experiment is shown in the porometer and sto-

matal measurements. The aperture was practically the same under the three qualities of light in each comparison. It also differed very little in the different tests and was the same in leaves E and F at the same temperature. The effect of filtration through a chlorophyll solution in reducing the efficiency of light for promotion of stomatal opening is very marked. Still more striking effects have been observed with high light intensity and a denser chlorophyll screen. It has been reported (**12**) that sunlight passed through a certain chlorophyll solution failed to produce any stomatal opening in Zebrina, although, when passed through a neutral filter which reduced the light intensity more than the chlorophyll did but without altering light quality, transmission was sufficient to cause full opening.

In the literature on the relation of stomatal opening to light quality the only important results opposed to the photosynthesis theory are those of SIERP (**15**) and HARMS (**6**), who found red light to be regularly the least active. Their light source, a 500-watt incandescent lamp, has a very high emission of infrared compared with a fluorescent one, and they measured only total radiation. Sierp states that, in combination with 1 cm. of 6% $CuSO_4$ solution, none of their color filters transmitted radiation containing more than 7% of infrared. This estimate is surprisingly low, but even if correct, it does not apply to the red light of their stomatal experiments, because in these they substituted a blue filter for the $CuSO_4$ solution when using their red one. The blue filter was used because it transmitted more red light, but, in that case, it probably also transmitted photosynthetically inactive far red and near infrared which $CuSO_4$ cuts out.

Moreover, in measuring radiation from a hot source, it is difficult to exclude the effect of long-wave infrared emitted by the heated bulb and accessory parts of the lamp. Since long-wave radiation passes through filters fairly freely irrespective of their transmission of visible light, it may constitute an important part of what gets through a very opaque filter such as the red one used by Sierp and Harms. To equalize the radiation, they combined photographic plates, as required, with the less opaque color filters. Since these plates block off some of the long-wave infrared, the combination would tend to yield a higher proportion of visible light than the red filter alone.

From all these considerations, it seems possible that Sierp's and Harms's red light may have been the least efficient because it contained the highest proportion of photosynthetically inactive extreme red and infrared. It is suggestive that PAETZ (**14**), who found the action of different colors of light on stomata to be red > blue > green, estimated light intensity by a quantitative method that excludes infrared.

CARBON DIOXIDE

The new factor that was varied in the following experiments was the supply of carbon dioxide to the leaf, using supply to cover both the concentration of CO_2 in the air, and the rate of air flow.

EFFECTS OF CO_2-FREE AIR ON STOMATAL OPENING AND GAS EXCHANGE.—In addition to porometer measurements, respiration and photosynthesis rates were determined on leaves that had come to equilibrium in normal and CO_2-free air respectively at different light intensities. Respiration under such conditions was measured as the rate of output of CO_2 after darkening for five minutes, photosynthesis in CO_2-free air as the difference between this and the output in light.

Results obtained with three leaves are summarized in table II. The first experiment, leaf G, gave very wide opening in CO_2-free air at 45 fc light intensity—which as the next line in the table shows was the compensation point in normal air. The second experiment, leaf H, shows that fairly wide opening may occur in CO_2-free air in the dark. With the stomata slightly

TABLE II

EFFECT OF CO_2-FREE AIR ON STOMATA AT DIFFERENT LIGHT INTENSITIES.

Air flow: 35 l/hr.; v.p. of air: 12 mm. Hg; Leaf temperature: 24° ± 0.5° C.

Leaf	CO_2 conc. vol. %	Light intensity fc	Hours interval	Equilibrium		Mg. CO_2/dm²/h	
				Pore area (μ^2)	Mean diam. (μ)	Resp.	Photo.
G	0.03	(45)	0.0	8	4.4	....	....
	0.00	45	3.38	190	16.7	0.45	0.10
	0.03	45	3.50	5	3.7	0.45	0.45
	0.03	500	3.35	67	10.7	0.45	5.25
H	0.03	(0)	0.0	7	4.2	....	....
	0.00	0	3.50	63	10.4	0.80	....
	0.03	0	4.00	0	0.0	....	0
	0.00	0	2.00	0	0.0	0.47	0
	0.00	0	12.00	63	10.4	....	0
	0.00	30	1.00	87	11.7	0.80	< 0.10
	0.00	500	6.75	185	16.2	0.80	0.16
	0.00	0	14.00	49	9.6	0.80	0.00
I	0.03	1000	3.50	90	11.8	....	....
	0.00	0	1.00	47	9.4	....	....

open at the start the response to removal of CO_2 proceeded at about the same speed as with changes of light intensity, but with the stomata initially shut no trace of opening occurred within two hours. At the end of 14 hours, the same degree of opening had developed as in the former case but it is likely that this was initiated by some slight night opening, for, as Heath concluded, CO_2-free air probably has no effect if all the stomata are completely shut.

The comparison in leaf I of the effect of CO_2-free air with that of light is inadequate, because the period of exposure to the former was scarcely long enough to ensure equilibrium. The wider opening shown by leaf G in absence of light and CO_2 is probably more typical. It is comparable to that produced by light of 400 fc in normal air.

The following points may be noted regarding the few measurements of respiration and photosynthesis shown in the last two columns of the table. Comparison of G and H suggests one possible reason why leaves vary in their sensitivity to CO_2-free air as well as to light. Opening was slightly greater in the former at 45 fc than in the latter at 500. The much higher respiration rate of the latter might account for this. Other points are: respiration with stomata closed was 60% of that with stomata open (H), but was the same at a small as at a large aperture (G); respiration was the same in CO_2-free air as in normal air (H); only a small fraction, about one-fifth, of the CO_2 released by respiration was captured by photosynthesis in CO_2-free air (G and H), even with light of 500 fc.

This last finding exemplifies the fact that there can be no compensating light intensity in CO_2-free air. The CO_2 produced by respiration is free to diffuse either out of the leaf or to the chloroplasts, the relative rates depending largely on stomatal aperture on the one hand and light intensity (up to a point) and the photosynthetic efficiency of the chloroplasts on the other hand. Only in the presence of a certain concentration of CO_2 in the external air can photosynthesis compensate for respiration. The results of GABRIELSEN (**3, 4**) and HEATH (**8**) suggest that the minimum concentration of external CO_2 required for this is 0.005 to 0.01% at about 1000 fc, or possibly any higher, light intensity.

The relative effect of CO_2-free air on stomatal opening in presence and absence of chlorophyll tissue was the subject of the next experiment. On this occasion stomata were measured by Lloyd's method on strips taken from green and white areas of a variegated Pelargonium leaf. Twenty were measured on each strip. The leaf after being exposed to sunlight until its stomata approached their widest, was then placed in a stream of CO_2-free air under weaker light of 400 fc (table III). Opening increased in both white and green areas but much more in the white, where it increased three and one-half times. It rose from 30% to 77% of that in the green area and to 110% of what the latter had been in normal air. On darkening, both decreased somewhat, and also converged to the same size, which was equal to that of the green area in morning sunlight and normal air. This opening

TABLE III

RELATIVE EFFECT OF NORMAL AND CO_2-FREE AIR ON STOMATA OF WHITE AND GREEN TISSUE IN VARIOUS TREATMENTS.

Treatment	Time	Stomatal pore width μ		Width of white in % of green
		Green	White	
Normal air				
Sunlight	Until 8.00 A.M.	7.0 ± 0.43	2.2 ± 0.27	30
CO_2-free air				
Light 400 fc	Until 12.30 P.M.	10.0 ± 0.29	7.6 ± 0.16	76
Light 400 fc	2.00 P.M.	10.1 ± 0.20	7.7 ± 0.18	77
Darkness	4.00 P.M.	7.0 ± 0.34	6.7 ± 0.25	
Darkness	8.00 P.M.	7.0 ± 0.33	6.9 ± 0.19	99

was close to the peak opening of green areas in sunlight on the previous day. The 23% difference that remained between green and white region stomata in CO_2-free air in light must seemingly be due to photosynthetic reduction of respiratory CO_2 by the green mesophyll, since leaf temperature was not appreciably raised by the radiation and the stomata respond equally to absence of CO_2 in darkness.

These results show that stomata of white and green tissue respond in the same way to reduction in the concentration of CO_2 outside and inside the leaf, and that the difference between the two regions as regards the degree of opening in light is capable of being explained by the lower CO_2 concentration in the green tissue.

EFFECT OF VARYING THE RATE OF AIR FLOW.—One leaf (J) was exposed to two rates of air flow at each of two light intensities and another (K) to three rates of flow at a higher intensity. Table IV gives the measurements

TABLE IV

EFFECTS OF LIGHT INTENSITY AND RATE OF AIR FLOW ON PHOTOSYNTHESIS AND STOMATAL APERTURE AT EQUILIBRIUM.

Leaf chamber 12.3 × 2.6 cm.

Leaf	Light int. fc	Air flow l./hr.	Net Photo. mg. CO_2/dm.²/h.	% Reduction of CO_2 in air (0.03% = 100)	Pore area (μ^2)
J	240	50	2.54	5.7	44
	240	25	1.65	7.6	74
	420	50	4.43	10.0	80
	420	25	3.04	13.9	105
K	900	45	3.06	10.0	88
	900	50	3.40	10.3	88
	900	24	2.40	14.7	134

of photosynthesis and stomatal aperture, made when both had become constant and remained so for about an hour. There is added the amount of CO_2 taken out of the air in passing through the leaf chamber, expressed in per cent. of the entering concentration. The effect of the environmental factors can be judged strictly only in each individual leaf. At a constant rate of flow, increase of light intensity, of course, augmented both photosynthesis and stomatal aperture. At constant light intensity, reduction in rate of flow reduced photosynthesis but increased stomatal aperture. Both changes caused greater reduction in the CO_2 concentration of the air during its passage through the chamber. Halving the rate of air flow increased the removal of CO_2 by 33% to 42%, rising with the light intensity. The greatest relative increase in pore area occurred at the smallest aperture, where the internal concentration of CO_2 would fall farthest below the external.

The more immediate changes in photosynthesis rate following changes in flow were also measured at a single light intensity. By avoiding all unnecessary delay and altering the direction of change it was sought to pre-

TABLE V

IMMEDIATE EFFECT OF VARYING AIR FLOW ON RATE OF PHOTOSYNTHESIS. LIGHT INTENSITY 500 FC.

Rate of air flow, l/h	65	65	40	10	65	26
Net photosynthesis, mg. CO_2/dm.²/hr.	5.3	5.3	5.2	2.9	5.2	4.63
% Reduction in CO_2 concentration of air	10	10	16	37	10	21

vent any considerable movement of the stomata during the whole experiment. Table V gives the flow rates in the order used and below each the corresponding photosynthesis measurement and the per cent. reduction in the normal concentration of CO_2. It is evident that at low rates of air flow the CO_2 concentration outside and inside the leaf may be greatly reduced.

Another experiment deals with the effect of varying the rate of air flow on the time courses of 1/R and transpiration (fig. 5). A reduction in rate of flow produced an immediate drop in transpiration followed by a gradual rise as stomatal opening increased. Similarly an increase in flow produced an immediate rise and subsequent drop extending to about the original level. (In this case the transpiration rate declined more sharply than 1/R at the start, indicating that internal non-stomatal control was operating to some extent.) These results support those of HEATH and MILTHORPE (**10**) as to the effect of rate of air flow and CO_2 supply on stomatal opening.

EFFECT OF VARYING THE CARBON DIOXIDE CONTENT OF THE AIR FROM NORMAL DOWNWARD.—In the following experiments the air flow was kept constant at 64 l./hr. and the CO_2 concentration was regulated by mixing normal and CO_2-free air in definite proportions as estimated by means of flowmeters. All other conditions were kept constant. The effect on sto-

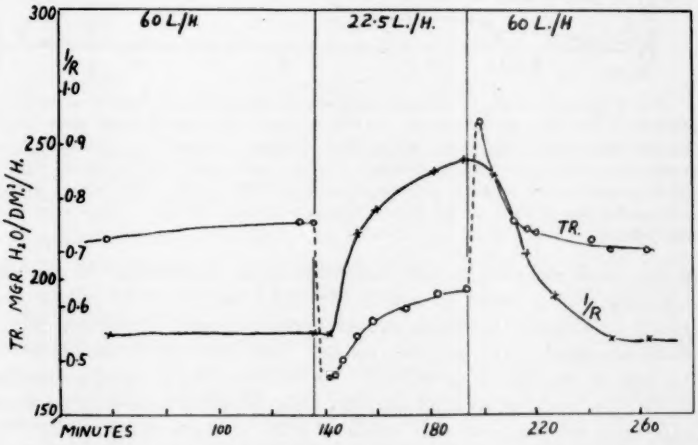


FIG. 5. Time courses of stomatal aperture (1/R) and of transpiration (TR) as affected by changes in the rate of air flow. L/H = liters per hour.

matal aperture at 50 and 400 fc was followed on the same leaf and the experiment partly repeated on the other leaves.

Figure 6 shows results obtained with one leaf, L. Curves 1a and 1b give the result of progressive reduction in the CO_2 content of the air under light of 50 fc, the former curve being pore area and the latter mean diameter of the stomata. Results with another leaf (M) were practically the same. Curve 2 shows the course of opening at 400 fc, first in normal air, and, after that had ceased, with one-eighth of the CO_2 removed. The powerful effect

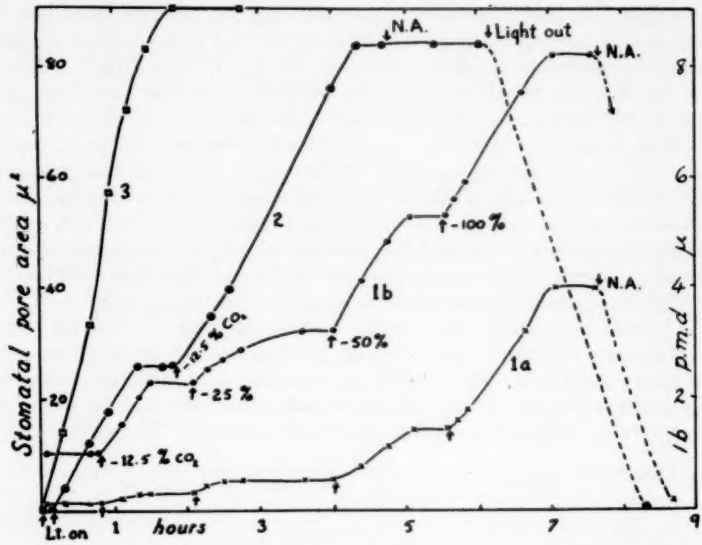


FIG. 6. Time courses of stomatal aperture as affected by fractional or complete removal of CO_2 from the air stream. Curves 1a (pore area) and 1b (pore mean diameter) represent a leaf kept under light of 50 fc starting in normal air, followed by progressive removal of the carbon dioxide. Curve 2 starts with application of light of 400 fc in normal air, followed later by removal of 12.5% of the CO_2. Curve 3 starts with application of light of 400 fc and complete removal of CO_2. NA = normal air supplied.

of this small reduction in CO_2 concentration is remarkable. It was also surprising that a return to normal air (NA) had no effect in this case, though subsequent darkening caused normal closure. Repetition of the whole experiment gave the same results. Reference will be made later to this lack of response to normal air. On the following morning removal of all the CO_2 from the air with the same light as before (curve 3) produced little more opening than that obtained with only one-eighth of the CO_2 taken out.

A striking contrast is here shown between the relationship of pore area to CO_2 reduction in light of 400 fc and 50 fc respectively. With the higher light the effect is greatest near the normal concentration of CO_2, with the lower light the effect is small at that concentration and increases up to complete removal of CO_2.

BEARING OF THE RESULTS ON THE RELATIONSHIP OF STOMATAL OPENING TO THE CONCENTRATION OF CO_2 INSIDE THE LEAF.—For purpose of comparison, the relation of pore area at equilibrium to the rate of photosynthesis at different light intensities in normal air and to the external concentration of CO_2 with a light intensity of 50 fc are shown together in figure 7. The

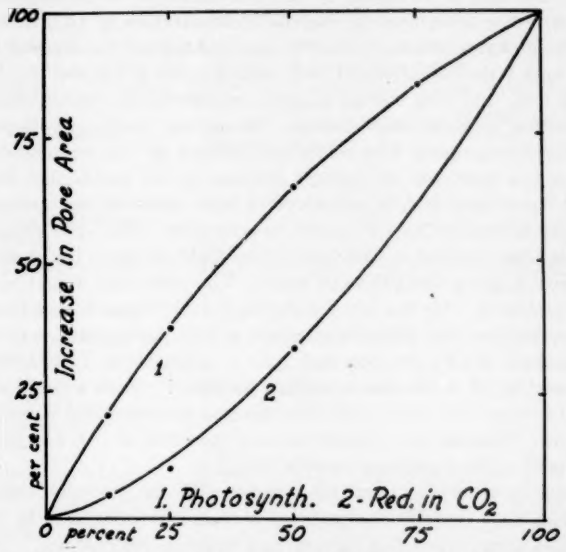


FIG. 7. Increase in pore area above that produced by light of 50 fc (compensation point) vs. (1) Rate of net photosynthesis as light is increased. (2) Reduction in CO_2 concentration of the air supplied. All values plotted in per cent. of the maximum.

zero point for both curves is the same, *viz.*, light of 50 fc and normal air. Curve 1 is an average of those in figure 4, II, excluding D_2. Curve 2 is an average of 1a in figure 6 and a repetition of the test. On the vertical axis, 100% represents about the same degree of opening in both cases relative to the widest possible opening, though the actual size of the stomata was greater in curve 1 (winter leaves) than in curve 2 (summer leaves).

Consider how the internal concentration of CO_2 is likely to vary in the course of the two curves. Both start at or near the compensation point in normal air, that is with the concentration of CO_2 in the intercellular spaces the same as in the atmosphere outside. In 1 the concentration inside falls

as the rate of fixation of CO_2 increases, in 2 it falls as the external concentration decreases. In 1, CO_2 is diffusing inward except at the start, in 2 it is diffusing outward. Widening of the stomata facilitates both processes, with the result that the drop in internal CO_2 concentration should be less than proportional to the rate of fixation of CO_2 (1) and more than proportional to the increase in outward gradient (2). The opposite directions of bending of the two curves is thus in harmony with the view that pore area depends in both cases on the level of CO_2 concentration within the leaf.

A corresponding curve for increase in pore area in relation to reduction of external CO_2 at 400 fc would rise steeply at first and then level off rapidly, reaching a maximum at perhaps one-third of normal CO_2 (**10**). A major factor governing the internal concentration of CO_2 at this light intensity is photosynthesis. As CO_2 supply becomes the limiting factor in this process, a decline in rate of CO_2 fixation tends to parallel the decline in external CO_2, and thus will increasingly counteract the direct effect of the latter on the internal concentration. Hence the distinctive shape of this curve too is in harmony with control of aperture by CO_2 concentration.

There is a good deal of scattered evidence in our results that the shapes of these curves may also be influenced to some extent by an increase in the sensitivity of stomata to CO_2 as its concentration falls. For example in a variegated leaf exposed to CO_2-free air and light of 400 fc the stomata were 30% wider in green areas than in white. The pore areas would be at least 2:1. Opening in CO_2-free air and darkness is the same in green and white tissue, so that the only differential action of light would seem to be the very small amount of CO_2 fixation that goes in green tissue in CO_2-free air—about one-fifth of respiration according to table I. Such a small reduction in CO_2 has very little effect when the internal concentration is near that of normal air. Possibly the relation between the effect of CO_2 and its concentration tends to be logarithmic within limits.

Evidences of protoplasmic regulation.—We have stressed findings and conclusions that simplify the problem of stomatal movement by reducing different external controlling factors, to a common internal one. But some of the behaviors indicate that the mechanism through which this single factor operates must be very complex, that is, protoplasmic rather than merely chemical or enzymatic.

The time curves of opening in response to a sudden change of light intensity or CO_2 concentration often show progression at an almost constant rate ending in an abrupt stop—quite unlike the course of a chemical or physical process proceeding to an equilibrium. Sometimes after remaining at rest for a while, the stomata suddenly renew their opening though no change has occurred in the environment (*e.g.*, fig. 2 A). A rarer phenomenon of movement that cannot be related to environmental change is oscillatory opening and closing that may continue for hours (Stälfelt, **16**; cf. **19**).

There is also indication of diurnal periodicity of movement at constant light intensity in CO_2-free air as has been reported previously for normal

air (GREGORY and PEARSE, 5). In carrying out the experiments with leaves L, M, and N, checks of the degree of opening in CO_2-free air with light of 400 fc were made at different times of day. The maximum and final apertures and the hours of day at which the exposures were begun and ended are given in table VI. The highest maxima, 90 and 85 μ^2, were obtained when exposure was started in the morning. When started at midday the maxima were 82 and 42 μ^2. Exposures started in late afternoon gave a maximum of 38 μ^2 in one case and showed a steady drop from an initial 43 μ^2 in another. The complete time curves show that in exposures started early, whether morning or midday, stomatal aperture started to fall from its maximum at

TABLE VI

STOMATAL MOVEMENT IN CO_2-FREE AIR UNDER LIGHT OF 400 FC AT DIFFERENT TIMES OF DAY. HOURS ARE IN STANDARD TIME.

Leaf	Pretreatment	Exposure started	Max. aperture μ^2	Time of max.	Aperture at end μ^2	Exposure ended
L	6 hrs 400 fc normal air. Then dark till stomata closed.	5.30 P.M.	38	7.30 P.M.	38	9.00 P.M.
L	All day 400 fc normal air. Then dark overnight.	9.00 A.M.	90	11.00 A.M.	discontinued	
M	Outdoors until exposure started.	12.30 P.M.	82	3.45 P.M.	26	9.45 P.M.
M	10 hrs 50 fc with progressive reduction from normal to zero CO_2.	4.50 P.M.	43	initial	21	11.40 P.M.
N	Outdoors until 10.20 A.M. Dark until stomata closed.	12 noon	42	3.00 P.M.	15	9.00 P.M.
N	Darkness and normal air overnight.	8.00 A.M.	85	11.00 A.M. until 4.00 P.M.	31	10.00 P.M.

3:30 to 4:30 P.M. and declined steadily thereafter as long as measurements were continued. It seems possible that the contrast, between the strong response of stomata to a small reduction of CO_2 concentration and the failure to respond to reversal of this change (fig. 5, curve 2), since it was observed in the morning, may have been associated with this internal rhythm.

No less significant than the occurrence of movements that are independent of any immediate external influence is the fact that all the stomata of a leaf display them simultaneously. Integration of activity is also exemplified in the response of stomata to shock, and the transmission of a shock stimulus across a leaf (WILLIAMS, 17). Though the movement of guard

cells depends on turgor change it appears that behind this there must be protoplasmic activity as definitely as in movements that depend directly on protoplasmic contraction.

Summary

Stomatal aperture at equilibrium is the same with different qualities of light when, at very different intensities, they produce a rate of photosynthesis that just balances respiration.

The relationship of stomatal opening to photosynthesis rate as compared with light intensity, and to the interaction of light intensity and the rate of supply of CO_2 to the leaf, is consistent with the view that the main action of light on stomata operates through photosynthetic reduction of CO_2 concentration within the leaf.

The effect of CO_2-free air in largely but not entirely reducing the superiority of opening in green as compared with white parts of a variegated leaf seems to favor the same view.

Though these findings tend to simplify the relation of stomatal movement to external factors, other phenomena, observed incidentally, point to a complex mechanism in the physiological response.

This research was supported by grants from the National Research Council of Canada.

DEPARTMENT OF PLANT PATHOLOGY
MACDONALD COLLEGE
QUEBEC, CANADA

LITERATURE CITED

1. ALVIM, P. DE T. Studies on the mechanism of stomatal behavior. Amer. Jour. Bot. **36**: 781–791. 1949.
2. FREELAND, R. O. Green pigments in guard cells. Abstract of paper presented at meeting of A.S.P.P. in Columbus, Ohio. **1950**.
3. GABRIELSEN, E. K. Threshold value of carbon dioxide concentration in photosynthesis of foliage leaves. Nature **161**: 138–139. **1948**.
4. GABRIELSEN, E. K. Photosynthesis in leaves at very low carbon dioxide concentrations. Nature **163**: 359. 1949.
5. GREGORY, F. G. and PEARSE, H. L. The effect on the behaviour of stomata of alternating periods of light and darkness of short duration. Ann. Bot. London N.S. **1**: 3–10. 1937.
6. HARMS, H. Beziehungen zwischen Stomataweite, Lichtstarke und Lichtfarbe. Planta **25**: 155–193. 1936.
7. HEATH, O. V. S. Control of stomatal movement by a reduction in the normal carbon dioxide content of the air. Nature **161**: 179–181. **1948**.
8. HEATH, O. V. S. Carbon dioxide in the intercellular spaces of leaves during photosynthesis. Nature **164**: 822–823. **1949**.

9. HEATH, O. V. S. The role of carbon dioxide in the light response of stomata. Pt. 1. Jour. Exptl. Bot. **1**: 29–62. 1950.
10. HEATH, O. V. S. and MILTHORPE, F. L. The role of carbon dioxide in the light response of stomata. Pt. 2. Jour. Exptl. Bot. **1**: 227–243. 1950.
11. PAETZ, K. W. Untersuchungen über die Zusammenhänge zwischen stomatärer Öffnungsweite und bekannten Instensitäten bestimmter Spectralbezirke. Planta **10**: 611–665. 1930.
12. SCARTH, G. W. Mechanism of the action of light and other factors on stomatal movement. Plant Physiol. **7**: 481–504. 1932.
13. SCARTH, G. W., LOEWY, A., and SHAW, M. Use of the infrared total absorption method for estimating the time course of photosynthesis and transpiration. Can. Jour. Res. C **26**: 94–107. 1948.
14. SHAW, M. Porometer technique for the estimation of mean diameter and diffusive capacity of stomata. Unpublished.
15. SIERP, H. Untersuchungen über die Öffnungsbewegungen der Stomata in verschiedenen Spektralbereichen. Flora **128**: 269–285. 1933.
16. STÄLFELT, M. A. Abhängigkeit der photischen Spaltöffnungsreaktionen von der Temperatur. Planta **6**: 183–191. 1925.
17. WILLIAMS, W. T. Studies in stomatal behaviour. I. Stomatal movement induced by heat-shock and transmission of such stimuli across the leaves of *Pelargonium zonale*. Ann. Bot. London **12**: 35–51. 1948.
18. WILSON, C. C. The effect of some environmental factors on the movements of guard cells. Plant Physiol. **23**: 5–37. 1948.

PHOTOSYNTHESIS AND METABOLISM OF ORGANIC ACIDS IN HIGHER PLANTS[1]

R. E. Stutz[2] and R. H. Burris

(WITH TWO FIGURES)

Received November 16, 1950

The importance of organic acids as photosynthetic intermediates, first suggested by Liebig, has been subject to speculation for over 100 years. The free organic acids or their phosphate esters still are included in the most recent schemes for photosynthetic assimilation of carbon dioxide (**8, 12**). Recent advances in our knowledge of the path of carbon in photosynthesis have been described so adequately (**3, 8, 9, 12**), that any extensive review of the subject here is unwarranted. Bennet-Clark (**2**) recently has reviewed the literature on the organic acids of plants.

Our early attempts to use higher plants for the biosynthesis of C^{14}-labeled organic acids (**7**) revealed that equilibrium in C^{14} distribution among the organic acids was not established even many hours after exposure to $C^{14}O_2$. The observed concentration of C^{14} in malic acid suggested the possible importance of this acid as an early photosynthetic product. Accordingly, the distribution of C^{14} among several organic acids from plants supplied $C^{14}O_2$ was investigated.

Materials and methods

Growth of plants and exposure to $C^{14}O_2$

Plants were grown in the greenhouse on sand or vermiculite and were supplied with nutrient solution 1 of Hoagland and Arnon (**13**). In some instances intact, mature plants were enclosed for treatment with $C^{14}O_2$; in other experiments small, intact plants were treated. Rhubarb leaves were removed from the plant and their petioles were immersed in water. Exposures of large plants to $C^{14}C_2$ were made in Pyrex glass cylinders (**7**, fig. 7); the CO_2 was generated internally by injecting 20% perchloric acid into a vial containing $BaC^{14}O_3$. Tobacco seedlings 1.5 to 2 inches high were grown in alundum extraction thimbles and exposed to $C^{14}O_2$ in stoppered 38 × 200 mm. Pyrex test tubes. Alternatively, these small plants were treated with $C^{14}O_2$ under a small bell jar type filtration chamber through which gas was circulated with a diaphragm pump.

Normally, after the plants were sealed in the chambers, they were illuminated to deplete CO_2 (and to generate any reducing agents active in photo-

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation. These results were presented at the meeting of the Botanical Society of America, Washington, D. C., September 1948.

[2] Present address: Argonne National Laboratory, Chicago 80, Illinois.

synthesis). Dark treated plants were darkened prior to supplying $C^{14}O_2$ to deplete any reducing agents. Other plants were preilluminated and then darkened and immediately exposed to $C^{14}O_2$. In tests on the interconversion of photosynthetic products, the plants were kept in the dark after a period of photosynthesis of $C^{14}O_2$.

Preparation of extracts

After exposure to $C^{14}O_2$, the leaves, or entire plant, were dropped quickly into liquid air. Petioles and midribs were removed from the leaves, and the frozen tissue was ground to a fine powder in an iron mortar; liquid air was added occasionally during the grinding. Five parts of powdered leaf tissue then were combined with two parts by weight of 0.5 *N* H_2SO_4, adjusted to pH 1–2 with a few drops of 7 *N* H_2SO_4, filtered if there were over 10 gm. of tissue and stored frozen in the refrigerator.

Celite 545 and plant extract were combined, in the proportion 14 gm. celite per 10 ml. extract, and were mixed thoroughly with solvent (1 volume : 1 volume, butanol-chloroform equilibrated with 0.5 *N* H_2SO_4) in a glass mortar. Some losses of organic acids were incurred in early experiments before the necessity for removing metal from the celite by thorough washing with HCl and water was realized. The slurry of celite and extract was packed in a column and extracted with 400 ml. acid equilibrated 1 : 1 butanol-chloroform mixture in 25- to 50-ml. portions. When tissue was limited, celite was combined directly with the ground, acidified tissue and the whole mixture was extracted with a correspondingly smaller volume of solvent.

Twenty-five ml. of water was added to the flask containing the non-aqueous solvent, and the dissolved acids were titrated with 0.1 *N* NaOH to the endpoint of phenol red. The non-aqueous phase then was removed with a separatory funnel and washed with two 20-ml. portions of water. The combined aqueous phases were concentrated to a fraction of a ml. by vacuum distillation and acidified with an amount of 7 *N* H_2SO_4 calculated to liberate the organic acids present as the sodium salts. The volume was made to 1 ml. with water. To the extract in the flask was added 1 gm. of silica gel (prepared according to Isherwood (**14**)), and extract and gel were mixed by passing them through an 80-mesh sieve. The gel was made into a slurry with about 2 ml. of a mixture of equal volumes tertiary-amyl alcohol and chloroform, which had been equilibrated with 1.0 M Na_2SO_4, and was placed on a small column. The column was washed with successive 0.5-ml. portions of the tertiary-amyl alcohol–chloroform mixture until the volume of extract totalled 5 to 10 ml. This extract was stored in the cold room if analytical separation was delayed.

Preparation of columns

Analytical separations were made on 18-mm.-inside-diameter columns with 3 gm. of silica gel or on 8-mm. columns with 1 gm. of silica gel. The

silica gel was mixed with 1 ml. 0.5 *N* H_2SO_4 per gram of gel (as the gel dried out over P_2O_5 the amount of 0.5 *N* H_2SO_4 had to be increased at intervals until 1.16 ml. were used per gm. of gel), and the powder was passed through an 80-mesh-per-inch bronze wire sieve to assure complete mixing. The powder was made into a slurry with some of the solvent to be used in developing the column. The slurry was poured carefully into a column without forming bubbles and residual gel was washed in with additional solvent. The gel was allowed to settle and the column to drain; the column shrinks about 2 cm. after the solvent drains. Gel adhering to the glass above the packed column was removed with cotton wads. A tight-fitting disk of filter paper was placed on top of the column and the column was compressed until shortened 1.0 to 1.5 cm. The column was filled with solvent and connected to the air line; the pressure was increased gradually to the operating point. After about 60% of the solvent had passed through, the pressure was lowered gradually to avoid cracking. Unless cracked or dehydrated, the columns may be stored (filled with solvent), and reused for several separations.

Analytical separations

For separation, the organic acids in tertiary-amyl alcohol–chloroform mixture were pipetted onto the column; 1 ml. was used per gm. of silica gel in the column. Isherwood (**14**) rates the capacity of a 3-gm. column at 10 mg. of mixed acids; we have separated effectively as much as 16 mg. of acids on a 1-gm. column. The solution of acids was forced slowly into the column with a pressure of 5 lbs. per square inch; just as the top of the column became dry, the pressure was released and a small portion of the solvent (usually 30–35 volume per cent. butanol in chloroform equilibrated with 0.5 *N* H_2SO_4) was added. Two or three more small portions of solvent were used to wash down the sides of the tube and to force the sample into the column. The column tube was half filled with solvent, connected to a solvent reservoir, and the operating pressure was applied above the solvent in the reservoir. Fractions of effluent (usually 0.7–2.0 ml.) were collected with an automatic fraction collector.

To each tube from the fraction collector 2 to 3 ml. of CO_2-free water and one drop of 0.01% phenol red were added. The acid was titrated with 0.1 *N* $Ba(OH)_2$ delivered from a 1-ml. burette. The tip of the burette was submerged and the contents of the tube were mixed with CO_2-free air. The titration of each tube was plotted as a step on a bar graph; figure 1 represents a typical separation and indicates the resolution of organic acids which may be expected on a silica gel column. Malic acid always was present in the plant materials used and served as a convenient reference point in identifying other acids. The positions of known acids were mapped. Acids separated included succinic, oxalic, glycolic, malic, citric, and isocitric acids. The "succinic acid fraction" may include fumaric, pyruvic and acetic acids and esters of other acids, when 30 or 35% butanol in chloroform is used for the developing solvent. Under these conditions aconitic acid is

not separated well from oxalic acid. (Authentic oxalic acid appears at the position designated in figure 1, and material recovered from natural products at this position precipitates as its calcium salt from aqueous solution. Crystalline calcium oxalate has been recovered from preparative columns by this method; it reacted stoichiometrically with potassium permanganate.) Glyceric and isocitric acids appear together in the effluent from the column. (The lactone of isocitric acid comes off the column at almost the same position as succinic acid, but the free acid derived from plant extracts or from hydrolysis of the lactone appears at the position shown in figure 1.) The other acids are separated effectively. A solvent such as 10% butanol in chloroform should resolve the possible mixtures in the "succinic acid fraction." The peak including isocitric acid is labeled "isocitric fraction" because of possible contamination with glyceric acid. We have not found

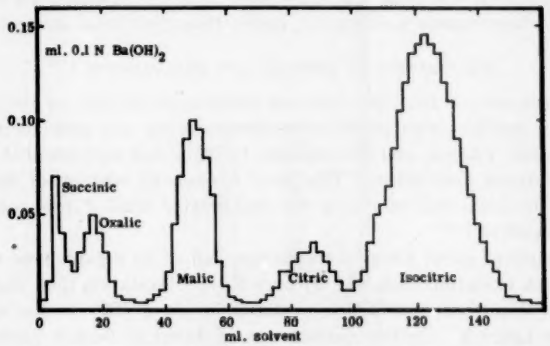


FIG. 1. Typical separation of organic acids from *Bryophyllum calycinum* on a silica gel column with 35 volume per cent. butanol in chloroform equilibrated with 0.5 *N* H_2SO_4 as solvent.

aconitic acid, so the oxalic peak is designated oxalic acid; the possibility of occasional contamination of oxalic by aconitic acid remains.

All tables list the mg. of each acid recovered, and these values are used in calculating the C^{14} as per cent. of the total in the acid fraction. The recovery of organic acids by the method employed is not complete, so the data should not be interpreted as strictly quantitative. The loss of organic acids occurs in their preliminary purification. In certain of the early experiments, the celite used was not thoroughly acid-washed; unwashed it contains heavy metal impurities, and added organic acids are not completely removed by the 1:1 butanol-chloroform mixture. In the second purification, the organic acids are placed on silica gel and eluted with a small volume of tertiary-amyl alcohol–chloroform mixture. This small volume of solvent does not remove the organic acids completely from the silica gel. The succinic and oxalic acid fractions are removed more completely

than malic and citric acids; in a second wash the ratio of malic and citric acids to succinic and oxalic acids will be higher than in the initial wash. ISHERWOOD (**14**) employed a more thorough extraction with the tertiary-amyl alcohol–chloroform mixture and reported almost complete recovery of organic acids. The small volume of solvent was used in our experiments to yield a more concentrated solution for addition to the analytical column and to insure that sufficient organic acids for a C^{14} analysis would be recovered. Although the data for amounts of organic acids present are not strictly quantitative, they indicate the approximate distribution of acids. As the specific activities of individual acids vary widely, there are probably few instances in which an entirely quantitative accounting would alter the order of the acids arranged according to their C^{14} content as per cent. of total in the acid fraction. The data of greatest importance in interpreting the sequence of formation and conversion of the compounds are their specific activities (counts/sec./mg. C), rather than their total amounts.

PREPARATION OF SAMPLES AND ANALYSIS FOR C^{14}

The contents of the tubes from an entire acid fraction, or the pure segment of a fraction, were pooled after titration; the non-aqueous phase was removed and washed, and the aqueous fraction was concentrated to a few ml. by vacuum distillation. This small volume of solution of the barium salts of the acids was stored in the refrigerator until it was sampled for measurement of C^{14}.

A sample of about 4 mg. of the barium salt of an organic acid was dried in a carbon oxidation flask (fig. 2) over P_2O_5. Oxidation then was effected with the Van Slyke and Folch oxidation mixture (**22**) in the apparatus shown in figure 2. Carbon dioxide was captured in freshly filtered, saturated $Ba(OH)_2$ to which NH_4Cl was added to suppress formation of bicarbonate (**11**). The centrifuge tube at the base of the Vigreaux column was loosened, and the column was rinsed with CO_2-free water. The centrifuge tube was capped and centrifuged. The precipitate was washed with CO_2-free water and centrifuged. The precipitate was suspended in methanol and centrifuged. A few drops of methanol were added to the $BaCO_3$ and a thick slurry was made. The slurry was spread evenly over a 2 sq. cm. circular area, inscribed with dividers on an aluminum dish, and was dried. The weight of the $BaCO_3$ was determined. Samples from a given experiment were all counted (thin window counter) at one time, and specific activities were calculated after appropriate correction for background, self-absorption and coincidence loss in counting. The error in counting the samples of low activity was less than 10%.

Results

PRELIMINARY EXPERIMENTS

In the initial experiment, *Bryophyllum calycinum* was exposed to $C^{14}O_2$ for 12 hours in the light and 12 hours in the dark. If a highly active tri-

carboxylic acid cycle were operative, one might anticipate that the C^{14} specific activities of the organic acids would approach equilibrium in 24 hours. As shown in table I a the specific activities differed widely. Malic acid had accumulated most of the total C^{14} and had the highest specific activity among the acids. Citric acid had a much higher specific activity than isocitric acid. In other early experiments with *B. calycinum*, an exposure to $C^{14}O_2$ for four hours in the light gave a distribution of C^{14} very similar to

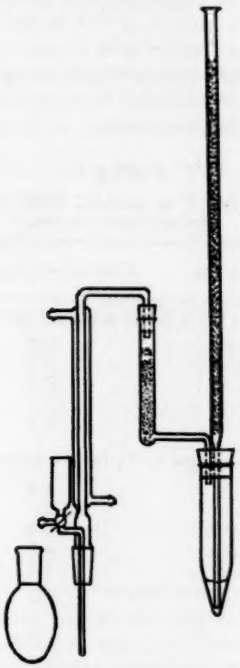


FIG. 2. Apparatus used in the wet oxidation of compounds to carbon dioxide. The oxidation flask has a 19/38 standard taper joint and has a capacity of about 45 ml. Air and oxidizing reagent are admitted through a 3-way stopcock. The tube preceding the 40-ml. centrifuge tube in the train contains 20-mesh granular zinc.

the 24-hour exposure; in a 2-hour exposure (table I b) the high percentage of the total activity in malic acid was accentuated.

At the time these first experiments were performed, there was much speculation on the possible importance of malic and succinic acids as early intermediates in photosynthesis (5). The abundance of C^{14} accumulated by malic acid in Bryophyllum was compatible with the idea that malic acid was a key compound in photosynthesis. Consequently, observations were

extended to other higher plants, and lengths of exposure to $C^{14}O_2$ were shortened to emphasize the differential distribution of the isotope. The description of subsequent experiments will be arranged according to the type of exposure to $C^{14}O_2$.

Exposure to $C^{14}O_2$ in light for 30 minutes

Several plant species were enclosed in glass chambers and exposed to $C^{14}O_2$ for 30 minutes in the light; prior to generating $C^{14}O_2$ the enclosed plants were illuminated to deplete the CO_2 in the chambers. *B. calycinum* (table II a) showed a distribution quite similar to that noted upon longer exposure to $C^{14}O_2$. Tomato (*Lycopersicon esculentum* Mill., variety John Baer) leaves (table II b) yielded only three organic acids in this separation. Malic acid had 92% of the radioactivity of the organic acid fraction and

TABLE I

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM LEAVES OF *Bryophyllum calycinum*

Organic acid	Acid, mg.	Counts/sec./mg. C	C^{14}, as % of total in acid fraction
a. Plants supplied $C^{14}O_2$ for 12 hours in light, 12 hours in dark			
Succinic fraction	0.62	4.6	0.9
Oxalic	0.50	2.1	0.2
Malic	5.27	59.0	89.0
Citric	0.71	19.0	4.1
Isocitric fraction	7.16	2.7	5.8
b. Plants supplied $C^{14}O_2$ for two hours in light			
Succinic fraction	1.03	3.8	1.8
Oxalic	0.10	2.7	0.8
Malic	1.97	114.0	93.2
Citric	1.21	4.1	2.2
Isocitric fraction	4.07	1.1	2.0

had about 10 times the specific activity of the other acids. Malic acid again exhibited its dominance in tobacco (*Nicotiana tabacum*, variety Comstock Spanish) (table II c). In barley (*Hordeum vulgare*, variety Wisconsin no. 38) (table II d) the differential in specific activities was less, but the malic acid still had the highest total and specific activity. The isocitric acid fraction had greater specific activity than succinic or citric acids; this differed from the isocitric acid fraction of Bryophyllum which contains an enormous reservoir of this acid. Rhubarb (*Rheum hybridum*, variety Victoria) exhibited even less differential than barley in specific activities of its organic acids (table II e). Oxalic acid was relatively inert, but succinic and citric acids were active metabolites.

Exposure to $C^{14}O_2$ in dark for 15 minutes

To determine the capacity of higher plants to fix $C^{14}O_2$ in the dark and to determine the distribution of C^{14} among the organic acids formed in the

dark, plants enclosed in glass jars were pre-illuminated to deplete CO_2 and then were exposed in the dark to $C^{14}O_2$ generated within the chambers. After 15 minutes exposure to $C^{14}O_2$, the plants were harvested rapidly in a hood in subdued light.

The data in table III a indicate that in Bryophyllum, malic acid again carried over 91% of the C^{14} in the organic acid fraction. In specific activity it was even more dominant than it was when the plants fixed $C^{14}O_2$ in the light. The distribution of C^{14} among the other acids was not greatly differ-

TABLE II

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM LEAVES

Organic acid	Acid, mg.	Counts/sec./mg. C	C^{14}, as % of total in acid fraction
a. *Bryophyllum calycinum* supplied $C^{14}O_2$ for 30 minutes in light			
Succinic fraction	0.98	3.0	1.8
Oxalic	0.29	....	....
Malic	4.07	40.6	89.2
Citric	2.31	3.8	4.9
Isocitric fraction	10.00	0.7	4.1
b. Tomato supplied $C^{14}O_2$ for 30 minutes in light			
Succinic fraction	1.15	8.8	1.8
Malic	6.66	90.6	92.3
Citric	3.80	9.6	5.9
c. Tobacco supplied $C^{14}O_2$ for 30 minutes in light			
Succinic fraction	1.18	8.1	2.4
Oxalic	0.26	6.1	0.3
Malic	10.10	43.0	95.3
Citric	1.23	7.3	2.0
d. Barley supplied $C^{14}O_2$ for 30 minutes in light			
Succinic fraction	1.97	55.0	10.3
Oxalic	0.45	16.2	0.5
Malic	4.26	233.0	83.5
Citric	1.09	41.7	4.0
Isocitric fraction	0.22	90.7	1.7
e. Rhubarb supplied $C^{14}O_2$ for 30 minutes in light			
Succinic fraction	0.39	325.0	15.9
Oxalic	0.53	136.0	6.0
Malic	0.59	820.0	53.7
Citric	0.82	254.0	24.4

ent from that in the light; isocitric acid was more favored relative to citric in the dark than in the light. The leaves from tomato (table III b) contained glycolic acid, and it ranked third in total and specific activity. Although malic acid had the highest specific activity, it was only about 2.5 times that of citric acid, whereas in the light the difference was almost tenfold. Mechanisms for formation of citric acid in tomato leaves apparently are favored in the dark relative to the light. This same response was apparent with tobacco, barley and rhubarb. Tobacco (table III c) was notable

for the higher specific activity of citric than malic acid. The total C^{14} in malic acid was much higher than in citric acid, however. Barley, representative of a plant which has a very small reservoir of organic acids, exhibited a distribution of C^{14} fixed in the dark which was quite different from that of the other plants examined (table III d). Malic acid contained less than

TABLE III

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM THE LEAVES OF PLANTS PREILLUMINATED THEN SUPPLIED $C^{14}O_2$ FOR 15 MINUTES IN THE DARK

Organic acid	Acid, mg.	Counts/sec./mg. C	C^{14}, as % of total in acid fraction
	a. *Bryophyllum calycinum*		
Succinic fraction	0.77	0.9	0.8
Oxalic	1.24	1.7	1.6
Malic	2.12	42.6	91.3
Citric	1.53	1.1	1.7
Isocitric fraction	6.23	0.7	4.6
	b. Tomato		
Succinic fraction	1.36	1.1	1.7
Oxalic	0.41	0.2	0.1
Glycolic	1.63	1.4	2.0
Malic	8.40	10.1	84.8
Citric	2.62	4.0	11.2
Isocitric fraction	0.29	0.8	0.2
	c. Tobacco		
Succinic fraction	0.98	2.0	2.5
Oxalic	0.16	1.5	0.2
Malic	13.70	5.7	87.5
Citric	0.98	8.5	9.8
	d. Barley		
Succinic fraction	2.18	58.2	42.7
Oxalic	0.19	6.1	0.3
Malic	3.74	42.6	47.5
Citric	1.20	23.4	8.7
Isocitric fraction	0.09	27.2	0.8
	e. Rhubarb		
Succinic fraction	0.97	3.0	4.7
Oxalic	0.50	0.3	0.2
Malic	6.70	6.1	57.0
Citric	5.05	5.1	38.1

half the total C^{14} of the organic acid fraction and its specific activity was exceeded by that of the "succinic acid fraction." The specific activities of citric and isocitric acids were relatively high. The fixation of $C^{14}O_2$ in the dark by rhubarb (table III e) yielded citric acid approaching malic acid both in total and specific activity. The shift in distribution between light and dark treatments was less pronounced than with most plants tested. Oxalic acid formed in the dark had very low C^{14} specific activity.

Exposure of depleted plants to $C^{14}O_2$ in dark for four hours

In the dark experiments described, the plants were illuminated until the time of addition of $C^{14}O_2$; one would expect that stored reducing compounds would be primarily responsible for the reduction of $C^{14}O_2$ assimilated in the dark. To find the distribution of C^{14} following "respiratory fixation" of $C^{14}O_2$, reducing compounds were depleted by darkening the plant for an hour before adding $C^{14}O_2$; the plants were kept in the dark for four hours after addition of the isotope before they were harvested. It is apparent from table IV that most of the C^{14} fixed in the organic acid fraction of tobacco and barley was incorporated into malic acid. Succinic acid had the next greatest amount of C^{14} in each instance. Oxalic acid from tobacco was inactive but from barley had a rather high specific activity. The specific

TABLE IV

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FROM THE LEAVES OF PLANTS DEPLETED IN THE DARK AND THEN SUPPLIED $C^{14}O_2$ FOR FOUR HOURS IN THE DARK

Organic acid	Acid, mg.	Counts/sec./mg. C	C^{14}, as % of total in acid fraction
	a. Tobacco		
Succinic fraction	1.28	0.6	3.7
Oxalic	0.42	0.0	0.0
Malic	15.10	1.4	93.9
Citric	0.48	1.1	2.4
	b. Barley		
Succinic fraction	0.94	2.3	8.3
Oxalic	0.12	4.5	1.4
Malic	3.60	7.0	85.6
Citric	0.34	3.8	4.6
Isocitric fraction	0.06	0.6	0.1

activity of citric acid was greater than half that of malic acid in both tobacco and barley.

Interconversion of acids

If a plant is allowed to photosynthesize $C^{14}O_2$ and then is placed in the dark for a period in the absence of $C^{14}O_2$, there should be an opportunity for the compounds containing C^{14} to interchange their carbon with other compounds by normal processes of degradation and synthesis occurring in the darkened plant. Under these conditions, the specific activity of malic acid would be expected to decrease if it were one of the early photoproducts, whereas citric acid and other acids might be expected to increase in specific activity at the expense of malic acid. This experimental technique appeared to be a simple and independent means for testing the observation of Vickery *et al.* (**25**) that malic acid in the darkened tobacco leaf is converted to citric acid.

Potted plants used to test the interconversion of compounds were sealed in glass jars and illuminated to deplete CO_2. Then $C^{14}O_2$ was generated within the chamber, and the plants were illuminated for 15 minutes. The plants were taken from the chamber, and alternate leaves were removed in dim light and immersed in liquid air. The remaining plant was covered with a cardboard drum and was not given additional $C^{14}O_2$; thus exposure to $C^{14}O_2$ was limited to the 15-minute period of illumination. After four hours in the dark, the remaining leaves on the plant were removed for analysis.

In table V are listed the analyses for Bryophyllum leaves exposed to $C^{14}O_2$ in the light and after four additional hours in the dark. Contrary to expectations, none of the organic acids decreased in specific activity during the 4-hour dark period. In fact, every organic acid isolated at least doubled

TABLE V

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FORMED IN THE LEAVES OF *Bryophyllum calycinum* IN THE LIGHT AND INTERCONVERTED IN THE DARK

Organic acid	Acid, mg.	Counts/sec./mg. C	C^{14}, as % of total in acid fraction
a. Plant supplied $C^{14}O_2$ for 15 minutes in light			
Succinic fraction	0.28	2.8	2.9
Oxalic	0.13	0.6	0.2
Malic	0.66	40.8	88.7
Citric	0.49	1.5	2.6
Isocitric fraction	2.50	0.7	5.6
b. Same plant after four additional hours in dark without $C^{14}O_2$			
Succinic fraction	0.47	7.9	1.2
Malic	3.19	97.0	90.8
Citric	1.57	9.8	4.7
Isocitric fraction	6.95	1.6	3.3

in specific activity. The most notable increase in specific activity was in citric acid; the increase was over sixfold. Very likely some of this citric acid originated from the highly active malic acid. The failure to recover oxalic acid after darkening is unexplained. Malic acid contained about 90% of the total C^{14} of the organic acid fraction.

Data from an experiment to test interconversion of organic acids in tobacco are given in table VI. The experiment was performed on a potted tobacco plant about 12 inches high. After exposure to $C^{14}O_2$ in the light for 15 minutes, 88% of the C^{14} of the organic acid fraction was in malic acid but the "isocitric fraction" had a higher specific activity. During the 4-hour dark period the specific activity of all the acids except the "isocitric fraction" increased; the most spectacular rises in activity occurred in oxalic and citric acids. As the amount of acid in the "isocitric fraction" was small, it could not account for the increase in specific activity of the other acids. Glycolic acid disappeared during the dark period.

TABLE VI

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FORMED IN THE LEAVES OF TOBACCO IN THE LIGHT AND INTERCONVERTED IN THE DARK

Organic acid	Acid, mg.	Counts/sec./mg. C	C^{14}, as % of total in acid fraction
a. Plant supplied $C^{14}O_2$ for 15 minutes in light			
Succinic fraction	0.83	11.6	3.4
Oxalic	0.06	2.4	0.1
Glycolic	0.11	7.2	0.2
Malic	14.80	18.9	88.2
Citric	0.88	11.8	3.4
Isocitric fraction	0.42	33.9	4.7
b. Same plant after four additional hours in dark without $C^{14}O_2$			
Succinic fraction	1.22	21.9	4.5
Oxalic	0.12	15.8	0.2
Malic	15.30	37.9	85.5
Citric	1.44	41.5	9.2
Isocitric fraction	0.17	22.8	0.6

The experiment with tobacco was repeated with the top 12 inches of two plants about 36 inches high; the cut ends were immersed in water. All acids (including the "isocitric fraction") except the "succinic fraction" increased in specific activity during the dark period. Again the glycolic acid disappeared in the darkened plant.

Barley, which has a lower content of organic acids and a more active respiratory metabolism than Bryophyllum and tobacco, differed from them also in its behavior in an interconversion experiment. Succinic, glycolic, malic, and isocitric acids all decreased in C^{14} specific activity during a dark period following photosynthesis of $C^{14}O_2$ (table VII). Only oxalic and citric acids increased in specific activity.

TABLE VII

DISTRIBUTION OF C^{14} IN ORGANIC ACIDS FORMED IN THE LEAVES OF BARLEY IN THE LIGHT AND INTERCONVERTED IN THE DARK

Organic acid	Acid, mg.	Counts/sec./mg. C	C^{14}, as % of total in acid fraction
a. Plants supplied $C^{14}O_2$ for 15 minutes in light			
Succinic fraction	0.80	13.6	5.3
Oxalic	0.14	14.9	0.7
Glycolic	0.85	6.6	2.1
Malic	3.13	66.3	88.8
Citric	0.62	9.7	2.7
Isocitric fraction	0.11	8.3	0.4
b. Plants from same pot after four additional hours in dark without $C^{14}O_2$			
Succinic fraction	0.96	10.6	6.0
Oxalic	0.25	45.8	4.4
Glycolic	0.25	3.1	0.4
Malic	4.16	36.1	78.3
Citric	0.50	36.2	9.9
Isocitric fraction	0.32	6.2	1.0

Discussion

The initial studies on biosynthesis of C^{14}-labeled compounds by higher plants revealed that even after several hours of photosynthesis of $C^{14}O_2$, the C^{14} was very unequally distributed among the organic acids. The early proposal by BENSON *et al.* (**5**) of a 4-carbon acid cycle in photosynthesis lent interest to the observation that malic acid regularly had the highest total and specific activity among the organic acids recovered. Subsequent work by BASSHAM *et al.* (**1**) has indicated that the 4-carbon acids may not function in the direct pathway of photosynthesis. The data presented here perhaps are of greater interest in respect to organic acid metabolism of plants than in respect to photosynthesis.

The distribution of C^{14} among the organic acids from leaves of Bryophyllum was very similar whether the plants were harvested two or 24 hours after exposure to $C^{14}O_2$ (table I). The $C^{14}O_2$ generated in the chamber was undoubtedly exhausted within an hour. The increase in activity of individual acids with time was largely at the expense of the malic acid or other compounds (*e.g.*, phosphorylated intermediates) not measured in our experiments. The lack of equilibration of C^{14} between malic and isocitric acid could be attributed to the great quantity of isocitric acid present. This explanation does not suffice for succinic acid, for the succinic acid is present in small quantities but has a far lower specific activity than malic acid even after 24 hours. If a tricarboxylic acid cycle functions in Bryophyllum, it is sluggish. A tricarboxylic acid cycle of greater activity may operate in plants other than Bryophyllum; for example, our tests with barley and rhubarb showed a more rapid approach to equal distribution of C^{14} among their organic acids. Even in these plants it is doubtful that the tricarboxylic acid cycle is of importance in photosynthesis, *per se*.

Several species of plants exposed to $C^{14}O_2$ in the light for a period of 30 minutes (table II) exhibited a remarkable similarity in the distribution of C^{14} among their organic acids. Apparently the organic acids are metabolized in a common manner in the various plants. In every instance, malic acid had the highest specific activity and the highest total activity. This consistently high activity of malic acid confirms it as the key organic acid in the metabolism of many plants. The extensive studies of VICKERY, PUCHER, and coworkers (**17, 25, 26**) also have led to the conclusion that malic acid is metabolically most active of the organic acids normally analyzed. Oxalic acid occupies the position of the least active acid.

As in the earlier experiments, succinic acid was much less active than malic acid but comparable to citric acid. This is compatible with, but not proof of, its formation from citric acid via a tricarboxylic acid cycle rather than directly from reduction of malate as early postulated by BENSON *et al.* (**5**). The relatively low activity of succinic acid indicates that the later view of CALVIN (**8**) is likely correct, that succinic acid is not directly functional in the cycle of photosynthesis but arises through side reactions.

The data obtained with Bryophyllum are interesting in relation to the plant's diurnal cycle of acidity. WOLF (**27**) reported that a high pCO_2 inhibits the loss of acidity from the Crassulaceae in the light. BONNER and BONNER (**6**) extended his observation and attributed the increased formation of acid at night to a lowered temperature and an enhanced pCO_2 within the tissues. They suggested the Wood and Werkman reaction as the most logical pathway for CO_2 fixation. THURLOW and BONNER (**21**), with the aid of C^{14}, established that leaves of Bryophyllum assimilated CO_2 in the dark. Analyses indicate that changes in the concentration of malic acid are chiefly responsible for the diurnal variations in the acidity of Bryophyllum (**18**). VENNESLAND (**24**) has found oxalacetic acid carboxylase to be widespread in the plant kingdom, but has reported no tests for its presence in the Crassulaceae. All the oxalacetic acid carboxylase preparations also were active in the reversible oxidative decarboxylation of malic acid (**10**). Our results on CO_2 fixation in the dark certainly are compatible with the primary formation of malic acid, most likely via oxalacetic acid. Specific activities of the organic acids isolated after a short exposure to $C^{14}O_2$ indicate that the participation of CO_2 in the formation of citric and isocitric acids in Bryophyllum is a much slower process than in the formation of malic acid.

Accumulated information suggests that diurnal, reversible shifts from starch to malic acid account for changes in acidity of Bryophyllum. One might postulate that the following sequence of reactions occurs: starch undergoes phosphorolysis to glucose-1-phosphate which is converted to pyruvic acid by the usual glycolytic mechanism; the pyruvic acid is carboxylated to oxalacetic acid (reaction enhanced by high pCO_2), which is reduced to malic acid. The reverse could occur under the influence of light. Such a scheme, though obviously an oversimplification of the many enzymatic reactions occurring simultaneously in the leaf, represents a feasible mechanism for acidification and deacidification of succulents.

PUCHER *et al.* (**19**) have demonstrated that starch is synthesized in some quantity in Bryophyllum leaves harvested in the morning and placed in the dark. The failure of VARNER and BURRELL (**23**) to verify such extensive starch formation in the dark might arise from use by the two groups of experimenters of Bryophyllum leaves in different physiological states. Varner and Burrell have followed the assimilation of $C^{14}O_2$ by *B. calycinum* under a variety of conditions and have found that among the organic acids malic acid accumulates the greatest concentration of C^{14}.

The role of isocitric acid may be compared in Bryophyllum and barley (table II, a and d). Bryophyllum contained large quantities of isocitric acid and its specific activity was low. Barley contained little isocitric acid, but its specific activity was high. Assuming a tricarboxylic acid cycle is operative in these plants, the data for Bryophyllum would support the subordinant role of isocitric to citric acid in the tricarboxylic acid cycle (**15**,

16), whereas the higher specific activity of isocitric than of citric acid in barley would support its direct function in the cycle. However, there is a good possibility that glyceric acid contaminated the isocitric fraction from barley. Peaks for glyceric and isocitric acids are coincident in the separation procedure employed, so contamination with a small amount of highly active glyceric acid could enhance greatly the activity of the small isocitric acid fraction found in barley. On the other hand, there is little doubt that isocitric acid comprises virtually all the isocitric fraction in Bryophyllum.

Among the plants tested, equilibrium of C^{14} was most closely approached in the leaf blade of rhubarb. From this evidence, one could postulate the more active operation of a tricarboxylic acid cycle in rhubarb than in the other plants tested.

The dark fixation of $C^{14}O_2$ by plants which have been illuminated until supplied the isotope gives a distribution of C^{14} as shown in table III. The similarity in C^{14} distribution when $C^{14}O_2$ is fixed in the light or in the dark by preilluminated plants seems sufficient to warrant the conclusion that the illuminated plant stores energy that can be used in the dark to effect a reduction of CO_2 very much like that accomplished in the light. BENSON and CALVIN (4) have found that the distributions of C^{14} fixed in the dark by preilluminated algae and barley is similar to that when $C^{14}O_2$ is fixed in the light.

When plants have been depleted of reducing power by storage in the dark before exposure to $C^{14}O_2$, their uptake of C^{14} is much less active than is that of illuminated or preilluminated plants (table IV). The differences in specific activities among the acids formed by what is often termed respiratory fixation are less striking than the differences among acids formed during photosynthesis. BENSON and CALVIN (4) have observed that incorporation of C^{14} into glutamic, succinic and isocitric acids is suppressed in the light but that radioactivity appears in these acids in darkened plants.

VICKERY *et al.* (25) have presented good evidence that malic acid in darkened tobacco leaves is converted to citric acid. It seemed that one might check this point by allowing a plant to photosynthesize $C^{14}O_2$ for a short time and then by allowing it to interconvert its labeled and unlabeled acids in the dark. Definitive experimental results on the interconversion of malic and citric acids were not obtained, for all of the organic acids in Bryophyllum and all but isocitric acid in tobacco increased in specific activity during the dark period (tables V, VI). The small amount of isocitric acid in tobacco could account for but little of the increased activity of the other organic acids. PUCHER and VICKERY (20) have been unable to demonstrate the presence of significant quantities of isocitric acid in tobacco leaves. This fact, in combination with the unusually high specific activity of the isolated fraction, suggests that the "isocitric fraction" in tobacco may have been predominantly glyceric acid. From the observed increases in C^{14} specific activities of the organic acids while the plants were darkened, one must conclude that during photosynthesis some compound(s) other than the

organic acids isolated was synthesized with high specific activity and that this compound(s) was converted to organic acids during the dark period. The results of the interconversion experiments imply that none of the organic acids accounted for in the separations are particularly early products of photosynthesis, but are preceded by another compound(s) of much higher specific activity.

Among the organic acids of barley, only citric and oxalic acids showed an increase in specific activity during the dark period (table VII). The oxalic acid was particularly active, but the quantity present was so small that considerable error was attached to its analysis. It is interesting that the citric acid increased to the same specific activity as malic acid in the dark. It is not clear why barley behaves so differently from tobacco and Bryophyllum, but it may be associated with the fact that barley stores comparatively little organic acid. Precursors may be channeled more extensively into carbohydrate rather than into organic acid synthesis in barley.

During the dark period, glycolic acid disappeared from tobacco and was reduced over threefold in barley. This decrease of glycolic acid in the dark has been noted by BENSON and CALVIN (**4**) in Chlorella and barley. The glycolic acid in our experiments was never particularly high in specific activity, although it has been shown to be an early product of photosynthesis (**4**).

Summary

Plants were exposed to $C^{14}O_2$, their leaves were removed, and the distribution of C^{14} among the organic acids of the leaves was determined. Even after 24 hours, the C^{14} was far from equally distributed among the organic acids of *Bryophyllum calycinum*. If a tricarboxylic acid cycle is operative in this plant, it is sluggish.

In leaves of Bryophyllum, tomato, tobacco, barley, and rhubarb exposed to $C^{14}O_2$ in the light for 30 minutes, malic acid had the highest C^{14} specific activity and the highest total activity among the organic acids isolated. Malic acid appears to be a key compound in the organic acid metabolism of the plants examined.

Plants allowed to fix $C^{14}O_2$ in the dark following a period of illumination assimilated particularly high concentrations and total amounts of C^{14} into malic acid. Fixation in the dark, following depletion of the plants in the dark, also yielded malic acid of high activity.

Plants were exposed to $C^{14}O_2$ in the light for 15 minutes, part of the leaves were removed for analysis, and the rest of the plant was kept for four hours in the dark without $C^{14}O_2$. In the dark the specific activity of citric and oxalic acids increased in barley and the specific activity of the other organic acids decreased. During the dark period, the C^{14} specific activity of all organic acids in Bryophyllum and all but the isocitric acid fraction in tobacco increased. Evidently a precursor of high specific activity was formed in the light by Bryophyllum and tobacco and was converted

to organic acids in the dark. Thus the organic acids isolated are not first products of photosynthesis but are formed from precursors synthesized in earlier steps of the photosynthetic process.

DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY OF WISCONSIN
MADISON 6, WISCONSIN

LITERATURE CITED

1. BASSHAM, J. A., BENSON, A. A., and CALVIN, M. The path of carbon in photosynthesis. VIII. The role of malic acid. Jour. Biol. Chem. **185**: 781–787. 1950.
2. BENNET-CLARK, T. A. Organic acids of plants. Ann. Rev. Biochem. **18**: 639–654. 1949.
3. BENSON, A. A. and CALVIN, M. Carbon dioxide fixation by green plants. Ann. Rev. Plant Physiol. 25–42. 1950.
4. BENSON, A. A. and CALVIN, M. The path of carbon in photosynthesis. VII. Respiration and photosynthesis. Jour. Exptl. Bot. **1**: 63–68. 1950.
5. BENSON, A. A., CALVIN, M., HAAS, V. A., ARONOFF, S., HALL, A. G., BASSHAM, J. A., and WEIGL, J. W. C^{14} in photosynthesis. Photosynthesis in plants. 365–401. Iowa State College Press, Ames. 1949.
6. BONNER, W. and BONNER, J. The role of carbon dioxide in acid formation by succulent plants. Amer. Jour. Bot. **35**: 113–117. 1948.
7. BURRIS, R. H., WILSON, P. W., and STUTZ, R. E. Incorporation of isotopic carbon into compounds by biosynthesis. Bot. Gaz. **111**: 63–69. 1949.
8. CALVIN, M. The path of carbon in photosynthesis. VI. Jour. Chem. Education **26**: 639–657. 1949.
9. CALVIN, M., BASSHAM, J. A., and BENSON, A. A. Chemical transformations of carbon in photosynthesis. Federation Proc. **9**: 524–534. 1950.
10. CONN, E., VENNESLAND, BIRGIT, and KRAEMER, L. M. Distribution of a triphosphopyridine nucleotide-specific enzyme catalyzing the reversible oxidative decarboxylation of malic acid in higher plants. Arch. Biochem. **23**: 179–197. 1949.
11. DAUBEN, W. G., REID, J. C., and YANKWICH, P. E. Techniques in the use of carbon 14. Anal. Chem. **19**: 828–832. 1947.
12. FAGER, E. W., ROSENBERG, J. L., and GAFFRON, H. Intermediates in photosynthesis. Federation Proc. **9**: 535–542. 1950.
13. HOAGLAND, D. R. and ARNON, D. I. The water-culture method for growing plants without soil. California Agr. Expt. Sta., Circ. **347**. 1938.
14. ISHERWOOD, F. A. The determination and isolation of the organic acids in fruit. Biochem. Jour. **40**: 688–695. 1946.

15. Ogston, A. G. Interpretation of experiments on metabolic processes using isotopic tracer elements. Nature **162**: 963. 1948.
16. Potter, V. R. and Heidelberger, C. Biosynthesis of "asymmetric" citric acid: a substantiation of the Ogston concept. Nature **164**: 180. 1949.
17. Pucher, G. W., Leavenworth, C. S., Ginter, Wanda D., and Vickery, H. B. Studies in the metabolism of Crassulacean plants: Changes in the composition of *Bryophyllum calycinum* during growth. Plant Physiol. **22**: 1–19. 1947.
18. Pucher, G. W., Leavenworth, C. S., Ginter, Wanda D., and Vickery, H. B. Studies in the metabolism of Crassulacean plants: The diurnal variation in organic acid and starch content of *Bryophyllum calycinum*. Plant Physiol. **22**: 360–376. 1947.
19. Pucher, G. W., Leavenworth, C. S., Ginter, Wanda D., and Vickery, H. B. Studies in the metabolism of Crassulacean plants: The behavior of excised leaves of *Bryophyllum calycinum* during culture in water. Plant Physiol. **22**: 477–493. 1947.
20. Pucher, G. W. and Vickery, H. B. The metabolism of the organic acid of tobacco leaves. I. Effect of culture of excised leaves in solutions of organic acid salts. Jour. Biol. Chem. **178**: 557–575. 1949.
21. Thurlow, J. and Bonner, J. Fixation of atmospheric CO_2 in the dark by leaves of Bryophyllum. Arch. Biochem. **19**: 509–511. 1948.
22. Van Slyke, D. D. and Folch, J. Manometric carbon determination. Jour. Biol. Chem. **136**: 509–541. 1940.
23. Varner, J. E. and Burrell, R. C. Use of C^{14} in the study of the acid metabolism of *Bryophyllum calycinum*. Arch. Biochem. **25**: 280–287. 1950.
24. Vennesland, Birgit. The β-carboxylases of plants. II. The distribution of oxalacetic carboxylase in plant tissues. Jour. Biol. Chem. **178**: 591–597. 1949.
25. Vickery, H. B., Pucher, G. W., Wakeman, A. J., and Leavenworth, C. S. Chemical investigations of the tobacco plant. VI. Chemical changes that occur in leaves during culture in light and in darkness. Connecticut Agr. Expt. Sta., Bull. 399. 1937.
26. Vickery, H. B., Pucher, G. W., Wakeman, A. J., and Leavenworth, C. S. Chemical investigations of the rhubarb plant. Connecticut Agr. Expt. Sta., Bull. 424. 1939.
27. Wolf, J. Beiträge zur Kenntnis des Säuerstoffwechsels sukkulenter Crassulaceen. V. Mikrorespiratorische Untersuchungen an Blattgewebe von *Bryophyllum calycinum*. Planta **29**: 450–467. 1939.

THE EFFECTS OF SOME SPECIAL TREATMENTS IN THE DEGREENING OF FLORIDA ORANGES AS MEASURED BY RESPIRATION RATE

BERT E. ROPER[1] AND ERSTON V. MILLER

(WITH ONE FIGURE)

Received March 25, 1950

Introduction

For many years it has been customary to treat certain varieties of citrus fruits, especially early oranges, with ethylene gas for the purpose of removing the green color from the rinds. This process is referred to by the trade as "coloring" but should more properly be termed "degreening." That the term "coloring" is ill-chosen may be seen from the fact that though the maximum amount of yellow rind pigments (carotenoids) is not reached by the time that oranges are mature green, present degreening methods serve only to remove the green pigments (chlorophylls) and do not increase the yellow pigments already present (**20**). (A special process of adding natural vegetable dyes to the peel is commonly used today and fruits so treated must be stamped "color added.") The procedure now followed for degreening (**28**) is to stack field crates of oranges in rooms which are equipped with means for heating, introducing ethylene, maintaining proper humidity, and circulating fresh air. The fruit is kept at approximately 85° F (29.4° C), 80 to 100% relative humidity and is treated with ethylene in low concentrations. Under these conditions a fruit which is quite green may require as much as 60 hours to degreen whereas a fruit in which chlorophyll has begun to decompose while on the tree requires proportionately less time. This method is essentially the same as it was 20 years ago, with improvements being found only in construction of rooms and mechanical equipment.

The need for degreening arises solely from a consumer preference for "orange" colored oranges, for in Florida (**29**) and in other regions having semitropical and subtropical climates some of the early orange varieties mature before losing their green color. Citrus fruits of any variety growing as an "inside" crop on densely foliated trees often retain much of their greenness long after maturity. It is common knowledge (**12**) that "maturity" refers to a stage of development and "ripening" refers to the process by which a mature fruit becomes edible or desirable as food. Apples, bananas, and pears, which contain a starch reserve, may become mature before harvest but may not ripen until the action of enzymes and life processes have hydrolyzed the starches to sugars, produced a certain amount of softening, and effected other changes such as rendering bitter principles insoluble.

[1] Senior author's present address: Winter Garden Citrus Products Cooperative, Winter Garden, Florida.

Oranges are different in that they contain practically no starch, so that ripening processes are not accompanied by changes such as those just mentioned. Instead, while on the tree, fruits ripen by increasing the volume of juice and per cent. of total soluble solids (mainly sugars) and by decreasing the per cent. of acids. This process is stopped by picking. Thus, maturity and ripeness in oranges is synonymous and oranges must be of desirable eating quality at time of harvest in order to be regarded as mature. In other words, they must always be "tree-ripened" if they are ripe at all.

Although the ethylene treatment does not appreciably affect the sugars and acids in the orange, the conditions under which the fruits are held in the degreening rooms tend to accelerate some of the biological processes which may shorten the period of marketability. If the time of treatment with ethylene could be shortened it would be of advantage to producers and would result in fruit with better flavor and longer keeping quality. However, it was felt that before any marked changes can be made in the commercial practices it will be necessary to learn more regarding the fundamental physiological processes involved during the degreening of the oranges. This in brief, is the reason for undertaking these investigations. Because of the known stimulating effects of ethylene on the respiratory processes of fruits it seemed desirable to approach the present problem by making observations on the effects of various treatments on the rate of respiration in oranges and correlating the results with degreening.

Review of literature

Considerable research has been done on the precursors of the chlorophylls, the factors influencing the development of these pigments, and the mechanisms whereby photosynthesis occurs. However, very little has been said about the process by which the chlorophylls are destroyed in plants. GARDNER (**8**) demonstrated the presence of a chlorophyll-decomposing enzyme in the rinds of Satsuma oranges and NOACK (**21**) concluded that decomposition of the chlorophylls is a process involving oxidative catalysis. ROTHEMUND (**23**), on the other hand, in discussing autumnal decomposition of the chlorophylls in leaves, states that "chemical details of this process are not known."

Despite the lack of information regarding the fundamental nature of chlorophyll decomposition, progress has been made in the practical aspects of degreening oranges. DENNY (**4**) was among the early contributors to the subject, showing that ethylene was the active agent in the fumes of the kerosene stoves which were used for degreening citrus fruits at that time. Since then many studies on the effects of ethylene have been made but few have been concerned with the destruction of chlorophyll in citrus fruits. DUFRENOY (**7**) observed cytological changes in cells of orange peel degreened by treatment with ethylene gas. He noted a "fatty degeneration" of the chloroplasts with the formation of oil drops in which the yellow pigments dissolved.

One of the first noticed effects of ethylene on fruits was the stimulating effect on the respiratory rate. BAIER, RAMSEY, and collaborators (**1**), in a very comprehensive study of the whole process of degreening citrus fruits, have contributed valuable information on means for improving the construction and equipment of degreening rooms. However, data they obtained on rates of respiration were slightly lower than those found in the work now being reported and no shortening of degreening time was apparent in their experiments, possibly because fruits which they observed were in an atmosphere gradually increasing in carbon dioxide concentration. In a preliminary report, STAHL and CAIN (**24**) described equipment for measuring respiration rates of degreening oranges but stated only that oranges "showed marked increase in respiration while exposed to ethylene gas." The studies of HALLER, ROSE, LUTZ, and HARDING (**10**) were made in an atmosphere containing no CO_2, but the respiration rates found during ethylene treatments were considerably lower and required much longer to reach a maximum than those found in the present experiments. The response that the above-mentioned investigators obtained might be explained by the fact that their fruit was probably post-climacteric and had undergone certain changes prior to the experiment while degreening on the tree.

Materials and methods

The following three lines of approach were made in the development of this investigation: (a) treatment of the fruits with growth-modifying compounds; (b) treatments with certain volatile organic compounds prior to or during exposure to ethylene gas; and (c) exposure of the fruit to radio-frequency radiation with and without concurrent treatment with ethylene. In these experiments the fruits under test were placed in standard glass aquaria ($10 \times 12 \times 20$ inches). These aquaria were fitted with double-weight bottoms and ends in which holes were drilled for inlet and outlet tubes. The respired CO_2 was absorbed by the KOH in a specially-designed plexiglass (methylmethacrylate) funnel. A capillary tube of plexiglass was bent into the shape of a ring and placed over the above-mentioned funnel to serve both for flushing out the used KOH solution and for adding a fresh amount of this material.

Lids to the aquaria consisted of double-weight glass. These were held in place by recess frames which in turn were fitted to the metal flanges of the aquaria. Glass junctions were made gas-tight by use of a pliable, plastic weather-stripping called "Mortite." Wooden blocks were cemented onto the glass lids to permit removal when changing samples of fruit. In addition to this the aquaria joints received several coats of spar varnish.

No control of humidity in the chambers was considered necessary since it was observed to range between 95 and 100%, which is approximately that found in many commercial degreening rooms.

The apparatus used for supplying gases at atmospheric pressure was a modification of that first described by MAGNESS and DIEHL (**18**) and subse-

quently improved by other workers (**5, 9, 27**). The essential principles involved were as follows. (See figure 1.)

The oxygen to replace that used in each respiration chamber (D) was supplied from two glass cylinders (B) approximately 2½ inches in diameter and 28 inches in length. Each cylinder contained a smaller glass tube. Water was maintained at the tops of these smaller tubes by a siphon connected to the constant-level water source (A). When the CO_2 in the respiration chamber was absorbed in the KOH there occurred a momentary reduction in pressure. Oxygen then moved into the chamber to replace the absorbed CO_2. This caused water to be siphoned over to replace the oxygen, thus restoring equilibrium to the system. Constant level was maintained in "A" by allowing water to drop slowly into the funnel shown in

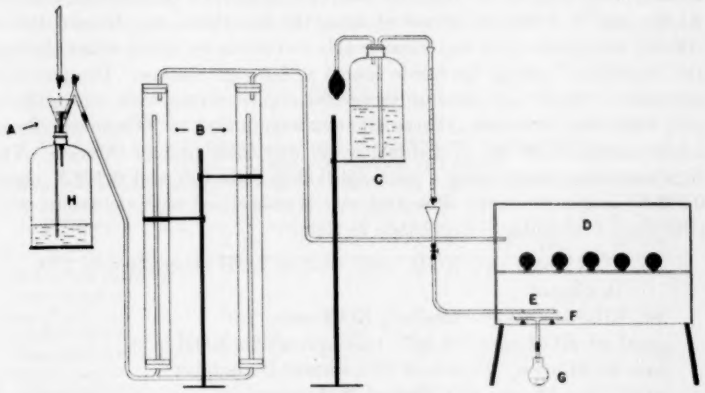


FIG. 1. Apparatus employed for studying respiration in oranges: A. Constant-level water source; B. Glass cylinders containing oxygen; C. Standard KOH; D. Respiration chamber; E. Sprinkler for flushing out old alkali and introducing a new supply; F. Plexiglass funnel for holding alkali; G. Flask for collecting spent alkali; H. Overflow water reservoir.

the diagram; when the level rose too high it drained into the overflow reservoir (H).

The procedure followed in most of the "runs" is described at this point and any deviations from this practice will be noted in the section concerned with the special treatment. The oranges were of the Parson Brown variety grown on sour rootstock near Ferndale, Florida. Fruits in Bruce box or bushel basket containers were shipped by Railway Express from Winter Garden, Florida, soon after picking. They were received four days later in Pittsburgh and immediately placed in a "walk-in" type refrigerator kept at 6° C (42.8° F). Twelve hours prior to the start of respiration measurements, oranges were removed to a covered container (not airtight but containing KOH pellets) in the constant temperature room, maintained at

32.2° C (90° F), so that a temperature equilibrium might be reached. Before starting the "run," fresh air was circulated into the chambers for several minutes by an electric fan. Ten oranges were then arranged in five rows of two each on glass racks and the chamber was closed and sealed. Immediately after closing the chamber, standard alkali was added and flushed in with about 10 ml. of water.

For the 0 to 3-, 3- to 6-, and 6- to 9-hour periods 25 ml. of approximately 2 *N* KOH was used. The amount added for the 9- to 24-hour period varied from 50 to 85 ml., depending upon the response found during the first nine hours. Several times in the course of the experiment the concentrations of the O_2 and CO_2 in the chambers were determined by analytical methods. Oxygen did not vary more than 1% from that in the outside air (average 20.85%) and only in one instance was CO_2 found in a measurable amount. At the end of a definite period of time, the absorbent was drained into a 100-ml. volumetric flask and made nearly to volume by water added through the "sprinkler," giving the inside funnel a thorough rinsing. The partially neutralized alkali was made up to volume and titrations were run in triplicate with 5-ml. aliquots. These aliquots were added to Erlenmeyer flasks which contained 50 ml. of distilled water and some barium chloride. The titrations were made using a phenolphthalein indicator and 0.242 *N* (later 0.247 *N*) sulphuric acid. The acid was standardized with potassium acid phthlate. Calculations were made as follows:

ml. KOH blank – ml. KOH "run" = ml. of KOH neutralized by CO_2 in aliquot
ml. KOH neut. × 20 = total ml. KOH neut.
total ml. KOH neut. × 0.242 = milliequivalents KOH neut.
m.e. KOH neut. / 2 = m.e. of CO_2 formed in chamber
m.e. CO_2 × 44 = mg. CO_2 formed in chamber
mg. CO_2 / (kg. fruit × hours) = CO_2 mg. per kg.-hr.

Results

Growth-modifying compounds applied as a spray

Since it has been claimed that the action of ethylene on plants is similar to that of auxins (**3**), it seemed logical in this work to attempt to substitute growth-modifying compounds for ethylene in degreening oranges.

The concentration of material sprayed on plants is known to be a large factor in determining the modifications caused. Since no recommendations concerning the concentrations most likely to increase respiratory rates of citrus fruits were available, sprays in four different concentrations were used. The growth-modifying compounds were dissolved in melted "carbowax" and this mixture was diluted with enough distilled water to make the final volume 2% carbowax. An aerosol type sprayer was used to apply the materials to the fruits just before the beginning of the respiration measurements. The treatments were made using 10, 100, 500, 1000 parts per mil-

lion of the compounds while the control lot was sprayed with a 2% solution of carbowax only. Respiration rates were measured only on the control and the lot treated with 500 p.p.m. spray. The remaining three lots were held in a covered aquarium containing an evaporated dish filled with KOH pellets to prevent accumulation of carbon dioxide. Observations were made on their rate of degreening.

Results with 2,4,6-trichlorophenoxyacetic acid, 3-indolebutyric acid, beta-naphthoxyacetic acid, and alpha-naphthaleneacetic acid showed that these compounds applied in this manner offered no promise as degreening agents, for at no concentrations of spray was there any color difference between the treated and control lots. The respiration measurements indicated that no major change in metabolic activities was caused by the treatments. In only one instance (during the first 3-hour period) did the control and treated lots differ by more than 6.5 mg. of CO_2 produced per kg.-hr. (10%). These data are found in table I.

TABLE I

EFFECT OF GROWTH-MODIFYING COMPOUNDS ON RESPIRATORY RATE OF IMMATURE FLORIDA ORANGES. EACH FIGURE REPRESENTS A SINGLE OBSERVATION ON 10 FRUITS.

Treatment	Mgs. of CO_2 per kg. per hr. during:				Total mgs. of CO_2 during 24 hrs.
	0–3 hrs.	3–6 hrs.	6–9 hrs.	9–24 hrs.	
(500 p.p.m. applied in aerosol spray)					
Control	46.6	55.4	55.4	53.9	1280.7
2,4,6,-Trichloro-phenoxyacetic acid	47.8	57.3	60.6	58.9	1380.6
Control	62.8	58.9	60.1	56.2	1388.4
3- Indolebutyric	48.1	58.0	58.9	51.9	1273.5
Control	54.2	55.8	60.1	56.3	1354.8
Beta-naphthoxy-acetic acid	52.3	....	57.3	50.6	....
Control	66.7	61.8	64.3	58.9	1461.9
Alpha-naphthalene-acetic acid	64.6	57.5	57.8	52.9	1333.2

GROWTH-MODIFYING COMPOUNDS APPLIED AS A VAPOR

Increased activity of the growth-modifying compounds was sought by introducing them into the chamber as a vapor at the start of a "run." To do this, a small amount of the material to be tested was placed in a vial fitted with a rubber stopper containing two capillary tubes. Through these tubes, air was forced over the compound into the respiration chamber. Increased volatility was obtained by heating the vial on a small hot plate, but even this produced only a slight loss of weight by the compounds (1 to 10 mg.). Data on the effect of the vapors of these compounds are summarized in table II.

Methyl alpha-naphthalene acetate produced almost no change in respiration rate and no color difference from that of the controls. With alpha-(*o*-chlorophenoxy)-propionic acid, the respiration rate was found to be slightly greater than the control lot but no color difference with fruits could be determined. Likewise cinnamic acid produced no effects upon the respiratory rate and no noticeable effect on degreening. However, the cinnamic acid was not treated with ultraviolet light to convert it from the trans to cis form as suggested by ZIMMERMAN and HITCHCOCK (**30**).

TREATMENT WITH CERTAIN VOLATILE ORGANIC COMPOUNDS

The effects of ethylene upon plant metabolism have been compared to those of the common anaesthetics (**13**). The next logical step in these studies therefore appeared to be a comparison of ethylene with other organic compounds, some of which are used as anaesthetics. Accordingly, experiments were set up in which three representative compounds, ethylene chlorohydrin, carbon tetrachloride, and chloroform, were tested for their effect upon degreening and respiration. The compounds were applied either as a pre-treatment prior to the respiration studies or as a supplemental treatment during respiration measurements. In the pre-treatment, the fruits were enclosed in a 50-pound lard can (capacity approximately 25 liters) and exposed to 0.2 to 0.5 ml. of the specific organic compound as it volatilized from a cheese cloth suspended from the sealed lid. When the volatile organic compound was applied directly to the respiration chamber the compound was likewise permitted to volatilize from cheese cloth. Ethylene gas was introduced into the chambers through the "sprinklers" used to add the alkali. The amount of gas was determined by adjusting the time required to displace the desired amount of water (about 8 ml.) from an inverted graduated cylinder and then allowing the gas to flow into the chamber for this length of time.

TABLE II

EFFECT OF VAPORS OF GROWTH-MODIFYING COMPOUNDS ON RESPIRATORY RATE OF IMMATURE FLORIDA ORANGES. EACH FIGURE REPRESENTS A SINGLE OBSERVATION ON 10 FRUITS.

Treatment	Mgs. of CO_2 per kg. per hr. during:				Total mgs. of CO_2 during 24 hrs.
	0–3 hrs.	3–6 hrs.	6–9 hrs.	9–24 hrs.	
(Vapors introduced into chamber)					
Control	68.7	65.6	67.3	54.9	1428.3
Methyl alpha-naphthalene acetate	70.7	66.1	64.3	53.8	1410.3
Control	48.4	55.8	50.5	48.9	1197.6
Alpha-(o-chloro-phenoxy)-propionic acid	62.0	57.4	59.0	50.8	1297.2
Control	56.5	62.2	58.7	52.5	1319.7
Cinnamic acid	57.8	58.9	60.5	49.9	1280.1

The increase in respiration rate of lots treated with ethylene was striking when compared to the rate of untreated controls. An ethylene-treated lot compared with a control lot showed that 53% more CO_2 was produced per kg. during the whole 24-hour period by the treated than by the controls and that respiration rate was 78% higher during the 9- to 24-hour period. If an average of the 9- to 24-hour periods of the eight control lots is taken as normal and compared to the average of the six ethylene treatments, an overall increase of 116% in respiration rate is noted. In every instance ethylene effected excellent degreening while the control fruit remained almost unchanged.

Ethylene chlorohydrin, a compound which has previously been shown to be physiologically active (**6**), was used in three different types of treatments. When this compound was introduced into the chamber in concentration of approximately 200 p.p.m. at the start of respiration measurements, a negligible increase in rate was seen and no degreening was produced. Ethylene chlorohydrin and ethylene were introduced simultaneously at the beginning of the respiration measurements on another lot and an increase in respiration rate during the first nine hours was observed. However, no color difference could be detected between fruits with this type of treatment and the ethylene controls. A 12-hour pre-treatment with ethylene chlorohydrin, followed by ethylene treatment, produced a 36% increase in the respiration rate during the first nine hours but the total CO_2 produced per kg. in the 24-hour period was only about 3% greater than the controls. The color of the ethylene controls was slightly better than the pre-treated lot.

Pre-treatments with the non-polar compounds, carbon tetrachloride and chloroform, were not effective in changing the rate of metabolism as measured by CO_2 production. Data for this series of experiments are summarized in table III.

Radio frequency radiation

Since the experiments thus far described gave no indication of the mechanism whereby degreening was being facilitated, another approach to the problem seemed desirable. Radiations have been shown to affect plant respiration by influencing many vital processes (**25**). It was hoped that, by applying energy of this type, a certain stimulation of the degreening mechanism of oranges might be obtained. The equipment employed in these experiments consisted of a Model CMD–4 Raytheon "Microtherm" which operated at a frequency of 2450 megacycles, had a maximum output of 125 watts, and was fitted with director C to produce an elliptical power pattern. The director was moved along the outside of the chamber (approximately four inches from the center of the oranges) and held opposite each fruit for one to two minutes per hour during the 0 to 9-hour period. Another type treatment was given by placing aluminum plates above and below the area occupied by the oranges in the chamber and "driving" them at 27.32 megacycles for 20 minutes per hour during the 0 to 9-hour period. This was done by using a Model No. 1000 "Meditherm" short wave diathermy unit

TABLE III

EFFECT OF VARIOUS ORGANIC COMPOUNDS ON RESPIRATORY RATE OF IMMATURE FLORIDA ORANGES. EACH FIGURE REPRESENTS A SINGLE OBSERVATION ON 10 FRUITS.

Treatment	Mgs. of CO_2 per kg. per hr. during:				Total mgs. of CO_2 during 24 hrs.
	0–3 hrs.	3–6 hrs.	6–9 hrs.	9–24 hrs.	
Control	46.7	65.4	70.4	64.2	1510.5
Ethylene	43.2	68.5	87.8	114.5	2316.0
Ethylene control	43.4	54.6	77.4	119.9	2324.7
Ethylene chlorohydrin	57.7	60.3	61.2	63.5	1490.1
Ethylene control	50.7	75.5	99.5	126.8	2579.1
Ethylene plus ethylene chlorohydrin	69.8	94.0	105.7	108.3	2433.0
Ethylene control	65.4	69.1	91.9	124.4	2545.2
Ethylene after 12 hours pretreatment with ethylene chlorohydrin	90.2	105.8	112.5	113.3	2625.0
Ethylene control	58.4	75.1	91.0	118.2	2446.5
Ethylene after 12 hours pretreatment with carbon tetrachloride	60.3	77.3	96.1	110.5	2358.6
*Ethylene control	57.8	74.3	95.1	119.5	2474.1
*Ethylene after 12 hours pretreatment with chloroform	66.6	88.0	102.4	115.1	2497.5

*Note: This last run was continued for 24 to 28 hours and the rate during this period was 101.1 for the ethylene control lot and 103.6 for the chloroform pretreated lot.

produced by the Medical Quartz Products Company. In each case, the power and current recorded were those indicated by the meter mounted in the equipment. After the first run was made, a 60-watt light bulb wired into the control chamber produced a rise in temperature equal to that caused by r-f energy inside the treatment chamber. The fruit itself showed a 1.5 to 2.0° C rise in temperature while under micro-wave treatment and a 3 to 6° C increase while under short-wave treatment, though surface temperature may have been even higher in the latter case.

The effect of microwaves on respiration during the 0 to 9-hour period was rather consistent. An increase in respiration of 33% was produced by an exposure of 10 minutes (one minute per orange) per hour at the 100% power level and a 20% increase was produced by 20 minutes per hour at the 65% power level. In neither of these experiments was degreening apparent, and it should be noted that, for the 24-hour period, the total respiration was only 14 and 8% above that of the controls. The treatment consisting of ethylene and microwaves produced a 27% greater respiration during the 0 to 9-hour period than did ethylene alone. However, the total respiration for the 24-hour period was only 7% above that of the controls and no difference in amount of degreening could be detected.

The data obtained from experiments using 11-meter treatments were

inconsistent. The exceptionally large increase in respiration of 81% during the 0 to 9-hour period in one lot was caused by a 20-minute per hour treatment at four amperes r-f current. The rate dropped rapidly after treatments were stopped so that the 24-hour average increase was only 53%. Since in one such experiment, fruits were severely burned (at 5 to 6 amps.) and results had to be discarded, it is likely that the tough dark green spots around the stem end of three oranges in this lot indicated that tissue damage had occurred and results were questionable. Also, ethylene plus a 20-minute per hour treatment at 2 amperes r-f current gave only a 10% increase in respiration during the 0 to 9-hour period and a total increase of only 4% for the 24-hour measurements. In neither case was any difference in amount of degreening in treated and control lots apparent. Data for this series of experiments are summarized in table IV.

TABLE IV

EFFECT OF RADIO-FREQUENCY RADIATIONS ON RESPIRATORY RATE OF IMMATURE FLORIDA ORANGES. EACH FIGURE REPRESENTS A SINGLE OBSERVATION ON 10 FRUITS.

Treatment	Mgs. of CO_2 per kg. per hr. during:				Total mgs. of CO_2 during 24 hours
	0–3 hrs.	3–6 hrs.	6–9 hrs.	9–24 hrs.	
Control	48.4	56.6	62.1	56.8	1353.3
10 min. of 12 cm. r-f per hr. for 0–9 hrs. at 100% power	60.8	81.0	81.0	58.3	1542.9
Control	72.4	85.7	76.9	61.1	1621.5
20 min. of 12 cm. r-f per hr. for 0–9 hrs. at 65% power	78.8	105.4	97.8	60.6	1755.0
Ethylene control	56.8	81.8	103.4	121.7	2551.5
Ethylene plus 20 min. of 12 cm. per hr. for 0–9 hrs. at 65% power	75.9	106.0	126.0	120.0	2723.7
Control	42.8	62.9	58.4	43.5	1144.8
Ethylene plus 20 min. of 11 meter r-f per hr. for 0–9 hrs. at 4 amps.	71.4	123.5	102.0	57.6	1754.7
Ethylene control	55.3	71.5	90.2	109.6	2295.0
Ethylene plus 20 min. of 11 meter r-f per hr. for 0–9 hrs. at 2 amps.	44.0	85.8	109.5	111.6	2391.9

Discussion

The conclusions to be drawn from the data gathered in this series of experiments are largely concerned with the mechanisms whereby degreening occurs in oranges and the way in which it is influenced by ethylene, for in no instance was any compound or treatment discovered which would produce a color change in less time than would ethylene alone. However, the oranges in these experiments showed a tendency to degreen with ethylene in somewhat less time than is normally required under commercial conditions.

It is possible that the O_2 and CO_2 content of the atmosphere was more accurately controlled under the experimental conditions than it is in commercial degreening rooms. Furthermore, a certain amount of delay between picking and degreening occurred as a result of the transportation between Florida and Pittsburgh and it is likely that degreening mechanisms were initiated by the fruits themselves during this period.

The experiments using growth-modifying compounds did not indicate that this type of treatment would be of use commercially nor did they produce any effects which could be correlated with degreening processes in fruits. However, it has been suggested that plant hormones are made more active by exposure to ethylene (**19**) and the influence of the growth-modifying compounds studied in these experiments should be further investigated by applying them in conjunction with ethylene treatments. This is especially true in the case of vapor treatments, since in this form the material should be better able to penetrate the cells of the plant and to reach the pigmented subepidermal layers. Since it was shown that compounds known to increase permeability did not alter the speed of degreening, one may conclude that ethylene alone diffuses into the plant parts rapidly enough and increases permeability sufficiently to allow gaseous exchange to occur as fast as is necessary for optimum degreening. The increase in respiration caused by ethylene chlorohydrin pre-treatment might be ascribed to its effect on enzymes, pH, or other metabolic factors. However, in describing the effects of ethylene chlorohydrin, CROCKER (**2**) says that because of the very multiplicity of factors influenced by this compound it is practically impossible to formulate a satisfactory explanation of the exact mechanism whereby it forces plant responses.

Although ethylene has been employed commercially for over 25 years the exact mechanism by which it induces ripening or coloring is still not entirely understood. Both E. M. HARVEY (**13**) and R. B. HARVEY (**14**) have reported that ethylene causes the simple soluble substances in plants to increase at the expense of the higher, soluble and insoluble forms. Increased activities of hydrolytic enzymes in pineapples (**22**), pears, peaches, and lemons (peel) (**11**), have also been demonstrated, so that this effect of ethylene is generally known. IVANOV, PROKOSHEV, and GABUNYA (**15**) went so far as to state that "in our opinion the role of ethylene may be reduced to the acceleration of the internal disintegration or partial autolysis of the living cells." However, they also showed that catalase activity was reduced in fruits treated with ethylene while the amount of peroxidase was increased by this compound. These effects on hydrolytic and oxidative enzymes are seen to be extremely important when associated with NOACK's (**21**) explanation of the biological decomposition of chlorophyll. He proposed that the first step in degreening is the proteolysis of the plastid stroma and this would seem to be confirmed by Dufrenoy's cytological observations (**7**). Thus it would seem that the increased activity of proteolytic enzymes is the first process speeded up by ethylene. This hastens the breakdown of the protective lipo-protein complex (**26**) of the stroma and enables chlorophyllase to form the phyllins

more rapidly. Also by causing the starch present in the plastid (**7**) to be hydrolyzed, material is supplied for the increased respiration which occurs, possibly because of the reduction in catalase. Noack further suggested that decomposition of chlorophyll occurs as a result of the oxidative action of hydrogen peroxide on the products of chlorophyll hydrolysis which are present in the form of their soluble alkali salts. Their oxidation is catalyzed by colloidal ferric hydroxide which is formed from the iron of the chloroplast. Since oranges have been shown not to contain oxidase (**17**) it seems likely that increased respiration is necessary to produce the H_2O_2 required for this oxidation, for the peroxidase present in oranges is probably more than sufficient to utilize all the H_2O_2 produced at normal respiration rates. However, it seems logical that when the respiration of fruits in a normal atmosphere was increased by irradiation during the 0 to 9-hour period H_2O_2 was not the limiting factor, but in this instance the increased respiration did not speed degreening because the hydrolytic enzymes had not broken down the plastids sufficiently to allow hydrolysis and oxidation of the chlorophylls.

From these observations the following hypothesis might be offered. The actions of ethylene and enzymes serve to hydrolyze the plastid stroma and produce materials which can be used in respiration. By this process, the chlorophylls are left unprotected, they are acted upon by chlorophyllase, and subsequently oxidized by H_2O_2 in the presence of a $Fe(OH)_3$ (or possibly a combination of $Fe(OH)_2$, $Cu(OH)_2$) catalyst. In this series of reactions the internal quality of the fruit is left unaltered because the activities are centered in the sub-epidermal layers of the peel.

The exact mechanism whereby ethylene acts upon the enzymes is not known. The idea of a co-enzyme relationship between ethylene and the oxidases as expressed by LYNCH (**16**) seems most feasible but should be extended to include the hydrolytic enzymes. It has been proposed by HARVEY (**14**) that the function of the ethylene double bond is to take on H^+ and OH^- ions and yield them easily to anhydrides. He also noted that the surface tension effects, solubility in aqueous and lipoid phases, as well as low molecular weight give this double bond compound properties not possessed by other compounds found in plants. Thus the reactions which cause degreening to occur are natural ones and the addition of ethylene is the most effective means known to make these processes occur at the maximum rate possible.

Summary

Using specially designed chambers, studies were made on the effects of various treatments on "degreening" and respiration of Florida oranges.

Several growth-modifying substances, applied either as an aerosol spray or as a vapor, did not appreciably affect the rate of degreening nor the rate of respiration. When certain volatile organic compounds were applied either as a pre-treatment or in conjunction with ethylene, no effect on degreening was noted, but there appeared to be a slight increase in respiration rate over that caused by ethylene alone. However this increased rate of respiration

was observed only during the 3-, 6-, and 9-hour determinations and did not appear in the 24-hour readings.

Treatments with radio-frequency radiations definitely accelerated respiration without affecting the rate of degreening.

An attempt was made to explain the observations recorded in these experiments by assembling what has been reported on the effects of ethylene on the physiology of the plant cell and especially the chloroplasts, thus postulating a theory which presents a comprehensive picture of the mechanisms involved in the loss of chlorophyll during the degreening of oranges.

Department of Biological Sciences
University of Pittsburgh
Pittsburgh 13, Pennsylvania

LITERATURE CITED

1. Baier, W. E. and Ramsey, H. J. Coloring citrus fruit. California Fruit Growers Exch. Res. and Field Depts., Los Angeles. 1932.
2. Crocker, William. Growth of plants. Reinhold Publishing Corp., New York. 459 pp. 1948.
3. Crocker, William, Hitchcock, A. E., and Zimmerman, P. W. Similarities in the effects of ethylene and the plant auxins. Contr. Boyce Thompson Inst. **7**: 231–248. 1935.
4. Denny, F. E. Hastening the coloration of lemons. Jour. Agric. Res. **27**: 757–769. 1924.
5. Denny, F. E. Respiration rate of plant tissue under conditions for the progressive partial depletion of the oxygen supply. Contr. Boyce Thompson Inst. **14**: 419–442. 1947.
6. Denny, F. E., Miller, L. P., and Guthrie, J. D. Enzyme activities of juices from potatoes treated with chemicals that break the rest period. Amer. Jour. Bot. **17**: 483–509. 1930.
7. Dufrenoy, J. A. Cytological study of water-soluble and fat-soluble constituents of citrus. Jour. Agric. Res. **38**: 411–429. 1929.
8. Gardner, W. A. The decomposition of chlorophyll in the rinds of satsuma oranges. Alabama Agric. Expt. Sta. Ann. Rpt. **37**: 16. 1926.
9. Haller, M. H. and Rose, D. H. Apparatus for determination of CO_2 and O_2 of respiration. Science N.S. **75**: 439–440. 1932.
10. Haller, M. H., Rose, D. H., Lutz, J. M., and Harding, P. L. Respiration of citrus fruits after harvest. Jour. Agric. Res. **71**: 327–359. 1945.
11. Hansen, E. The effect of ethylene on pectic changes in ripening fruits. Proc. Amer. Soc. Hort. Sci. **36**: 427–428. 1938.
12. Harding, P. L., Winston, J. R., and Fisher, D. F. Seasonal changes in Florida oranges. U. S. Dept. Agric., Tech. Bull. 753. 1940.
13. Harvey, E. M. Some effects of ethylene on the metabolism of plants. Bot. Gaz. **60**: 193–214. 1915.

14. HARVEY, R. B. Ethylene is a ripener of fruits and vegetables. Science N.S. **67**: 421–422. 1928.
15. IVANOV, N. N., PROKOSHEV, S. M., and GABUNYA, M. K. Biochemical changes in fruits under the influence of ethylene. Bull. Appl. Bot. Genet. Plant-Breed. (Russian) **25**: 223–278. 1930–31. (Eng. summary.)
16. LYNCH, L. J. A suggested coenzyme hypothesis for the ripening of fruits by ethylene gas treatment. Proc. Roy Soc. Queensland **47**: 18–24. 1936.
17. MCDERMOTT, F. A. Some data on the ripening of Florida oranges. Jour. Amer. Chem. Soc. **35**: 834–837. 1913.
18. MAGNESS, J. R. and DIEHL, H. C. Physiological studies on apples in storage. Jour. Agric. Res. **27**: 1–38. 1924.
19. MICHENER, H. D. Effects of ethylene on plant growth hormones. Science N.S. **82**: 551–552. 1935.
20. MILLER, E. V. and WINSTON, J. R. Investigation on the development of color in citrus fruits. Proc. Florida State Hort. Soc. Pages 87–90. 1939.
21. NOACK, KURT. Uber den biologishchen Abbau des Chlorophylls. Biochem. Zeit. **316**(1:2): 166–187. 1943.
22. REGEIMBAL, L. D. and HARVEY, R. B. The effect of ethylene on the enzymes of pineapple. Jour. Amer. Chem. Soc. **49**: 1117–1118. 1927.
23. ROTHEMUND, PAUL. Chlorophyll. In Medical physics (pp. 154–180), Otto Glasser, Editor. Year Book Publishers, Inc., Chicago. 1944.
24. STAHL, A. L. and CAIN, J. C. Cold storage studies of Florida citrus fruits. III. The relation of storage atmosphere to the keeping quality of citrus fruit in cold storage. Florida Agric. Expt. Sta., Bull. 316. 1937.
25. WEINTRAUB, R. L. Radiation and plant respiration. Bot. Rev. **10**: 383–459. 1944.
26. WEIER, ELLIOT. The structure of the chloroplast. Bot. Rev. **4**: 497–530. 1938.
27. WHITEMAN, T. M. and SCHOMER, H. A. Respiration and internal gas content of injured sweet-potato roots. Plant Physiol. **20**: 171–182. 1945.
28. WINSTON, J. R. Harvesting and handling citrus fruits in the Gulf States. U. S. Dept. Agric., Farmers' Bull. 1763. 1937.
29. WINSTON, J. R. and TILDEN, R. W. The coloring or degreening of mature citrus fruits with ethylene. U. S. Dept. Agric. (mimeographed).
30. ZIMMERMAN, P. W. and HITCHCOCK, A. E. Activation of cinnamic acid by ultra-violet light and the physiological activity of its emanations. Contr. Boyce Thompson Inst. **10**: 197–200. 1939.

COMPARISONS OF GROWTH BY TOBACCO AND SUNFLOWER TISSUE ON SYNTHETIC MEDIA CONTAINING VARIOUS SOURCES OF ORGANIC NITROGEN

ERICA M. FRANK, A. J. RIKER AND SARAH L. DYE

(WITH FOUR FIGURES)

Received June 10, 1950

Introduction

The importance of different organic and inorganic sources of nitrogen in plant growth has long been recognized. The main scope of the work on this subject has been reviewed by CHIBNALL (**2**) and by BÜRSTROM (**1**). Recent advances in plant tissue culture techniques have offered a new approach to the problems of nitrogen metabolism. By growing the tissues on synthetic media of known composition, it has appeared possible to determine directly which substances may be available to the plant tissue and which may have an inhibitory influence on its growth.

The early work on plant tissue culture was reviewed by WHITE (**15**) and GAUTHERET (**4**). The immediate background to this study, concerning the influence of temperature, pH, and composition of the medium on the growth of plant tissues *in vitro*, has been reported in papers by HILDEBRANDT, RIKER, and DUGGAR (**7, 9**) and HILDEBRANDT and RIKER (**5, 6**).

The effects of various organic and inorganic sources of nitrogen on the growth of excised sunflower tissue on synthetic media were investigated by RIKER and GUTSCHE (**13**). The majority of the organic compounds tested could not be utilized as a source of nitrogen. With sodium nitrate present as a source of nitrogen, these compounds caused progressive inhibition of growth with increase in concentration. A number of compounds, however, were available as sources of nitrogen; namely, urea, alanine, glutamic acid, aspartic acid, arginine, and asparagine. Tissues growing on alanine, glutamic acid and aspartic acid in the presence of 0.004 M sodium nitrate gave a distinctive type of growth curve. Growth was good to excellent at low and high concentrations of the amino acid, but was more or less completely inhibited at the 0.001 M level.

The purpose of this paper is to compare the growth of tobacco callus tissue with the growth of sunflower tissue on those organic nitrogen compounds which RIKER and GUTSCHE (**13**) found to be of special significance, and to attempt some explanation of the results obtained.

Materials and methods

The tissues under investigation were: bacteria-free sunflower tissue, isolated from secondary petiolar crown galls on *Helianthus annuus* L. var.

Giant Russian by HILDEBRANDT *et al.* (**7**), and tobacco callus tissue from the hybrid *Nicotiana glauca* Grah. ♀ × *N. langsdorffii* Weinm. ♂, originally isolated and supplied by Dr. P. R. White.

Six compounds were investigated as sources of nitrogen for tobacco tissue: sodium nitrate, ammonium succinate, urea, alanine, glutamic acid, and leucine. They were chosen from substances listed by RIKER and GUTSCHE (**13**), either because sunflower tissue growing on them gave distinctive growth reactions or because the substances had possible significance in nitrogen metabolism.

The reactions of the tissues to these substances were studied, not only by comparing the growth of tobacco tissue on tobacco medium with that of sunflower tissue on sunflower medium, as recorded by RIKER and GUTSCHE (**13**), but also, by comparing the growth of tobacco tissue with that of sunflower tissue growing on the tobacco medium at the same time under identical conditions.

The tissues were grown on a stock nitrogen-free synthetic medium to which the various nitrogen compounds were added, either alone or in combination with sodium nitrate. The cultural procedures used were essentially the same as those described by RIKER and GUTSCHE (**13**), except where noted. The stock medium for the sunflower tissue was prepared according to RIKER and GUTSCHE (**13**), and that for the tobacco tissue according to HILDEBRANDT *et al.* (**8**), except that glycine was omitted and calcium and potassium nitrate were replaced by equimolar quantities of calcium and potassium chloride. Pyridoxine hydrochloride was added to the tobacco medium in addition to thiamine hydrochloride to improve the growth of sunflower tissue on this medium.

Since concentration was found to be critical, the dilution series commonly used was 0.256, 0.064, 0.016, 0.004, 0.001, 0.00025, 0.00006, and 0.0 gram moles per liter, but for the less soluble amino acids the highest concentration used was 0.064 M. To prepare the dilution series, 20-ml. aliquots of double strength stock medium were placed in 6-oz. flint glass prescription bottles and the necessary amount of the nitrogen compound added, together with enough distilled water to make the volume up to 40 ml.

All glassware was cleaned in potassium dichromate–sulphuric acid, or sodium triphosphate cleaning solution. It was then soaked in hot tap water, rinsed, then soaked in distilled water for 30 minutes and again rinsed before using. No evidence of toxicity from residual chromium was observed.

The acidities of the basic medium and the solutions of the nitrogen compounds were adjusted in each series to pH 5.9 to 6.0 for the sunflower medium and to pH 5.7 to 5.8 for the tobacco medium. Additional replicates were set up at each concentration for checking the pH after autoclaving and at the end of the growing period.

Each series consisted of three bottles of medium at each concentration of the substance under investigation, plus three bottles of medium contain-

ing sodium nitrate as a nitrogen source. Growth on 0.008 M sodium nitrate was used as a standard of comparison in all cases. This control was necessary because of the variation in growth between individual series.

All the nitrogen compounds except urea were autoclaved with the medium. The urea was sterilized by filtering through porcelain or fritted glass. It was then added aseptically to the sterile basic medium.

Tissue transfers were made from stock cultures about six weeks old. Tissues were placed in a sterile Petri dish and cut into fragments weighing approximately 10 mg. The pieces were made smaller than those used by RIKER and GUTSCHE (**13**), in order to reduce carry-over of nitrogenous substances. This probably accounts both for the greater variation in the results obtained, and for the smaller final weight of the tissues. Four pieces of tissue were placed in each 6-oz. prescription bottle, all operations being carried out under aseptic conditions in a transfer chamber.

Cultures were incubated in a dark room, thermostatically controlled at approximately 26° C, with the humidity constant at about 40 to 50%. The air was kept in circulation by a low-speed fan. After six weeks, the tissues were removed from the medium. The adhering agar and surplus moisture were removed by gently flattening the tissues between pieces of filter paper. Each mass of tissue was weighed to the nearest centigram on a torsion balance. The average weight of the tissue pieces was used as a measure of growth on any given concentration of the nitrogen source.

In summarizing data, the conventions commonly used in bacteriology were employed. The amount of growth referred to that of the nitrate control in each case:

0 = no growth
± = a trace
\+ = poor growth, ¼ of control
++ = fair growth, ½ of control
+++ = good growth, ¾ of control
++++ = excellent growth, equal to or greater than control.

The variation of growth on 0.008 M nitrate in different series was so great that it was not found advisable to determine growth as a percentage of control for individual series. Instead, average weights of tissues from all trials were taken and compared with the average growth on the nitrate control.

Results

SUBSTANCES TESTED AS SOURCES OF NITROGEN

To determine the optimum concentration of sodium nitrate for the growth of tobacco tissue, it appeared necessary to compare the nitrate requirements with those of sunflower tissue and to obtain a standard for judging the amount of its growth on other substrates. Four trials were made with four bottles of medium at each concentration, covering a range of 0.005 to 0.064 M. Growth was good at concentrations of 0.002 to 0.016 M with the

optimum at 0.008 M. The average weight of tissues at the optimum concentration was 92 mg. after six weeks' growth at 26° C, as compared with 163 mg. obtained by HILDEBRANDT *et al.* (**9**), using the same basic medium. Weights of sunflower tissue on sunflower medium were also somewhat lower than those recorded by RIKER and GUTSCHE (**13**), but the reduction in growth was consistent. These differences were thought to be due (1) to the use of smaller tissue pieces as inoculum and (2) to drying the tissues with filter paper instead of using cotton gauze. However, variations in humidity of the culture room and changes in the tissue itself might have been partly responsible. The growth of tobacco tissue was only $\frac{1}{10}$ that obtained for sunflower by RIKER and GUTSCHE (**13**). This was to be expected since tobacco tissue is more compact than sunflower tissue and grows more slowly.

Five substances were tested as sole sources of nitrogen for tobacco tissue: ammonium succinate, urea, DL-alanine, L-glutamic acid and L-leucine.

Ammonium succinate permitted fair growth of sunflower tissue and was the only substance tested by RIKER and GUTSCHE (**13**) that gave evidence of the ammonium radical being utilized as a source of nitrogen. The tobacco tissue, like sunflower tissue, showed some growth on ammonium succinate at concentrations of 0.001 to 0.016 M but growth was nowhere greater than half that on the nitrate control. The optimum concentration for growth was 0.004 M, as compared with 0.016 M for sunflower tissue on the same medium (fig. 1).

Urea, which was such an excellent source of nitrogen for sunflower tissue, supported only fair growth of tobacco tissue on tobacco medium. Poor to fair growth of tobacco was generally obtained at concentrations of 0.00025 to 0.004 M, but it was always less than that on the nitrate control. There was no growth at concentrations above 0.016 M (table I). The optimum concentration for tobacco was 0.001 M, but for sunflower it was 0.016 M. In all cases much change either way from the optimum was unfavorable for growth (fig. 2).

DL-alanine, which in the absence of nitrate supported fairly good growth of sunflower tissue on sunflower medium, was also found to act as a source of nitrogen for tobacco tissue. Excellent growth was obtained at concentrations of 0.016 to 0.064 M, though it was never greater than that on the control. The optimum concentrate was 0.016 M as compared with 0.064 M for sunflower tissue on the same medium (**13**).

L-glutamic acid, which was found by LEONARD and BURRIS (**11**) to be connected with DL-alanine in plant tissues by a transamination reaction, had not previously been tested as a sole source of nitrogen for sunflower tissue—although it had been tested in the presence of sodium nitrate and found to act in much the same way as DL-alanine. Sunflower tissue, growing on sunflower medium, was found to utilize L-glutamic acid as a sole source of nitrogen, and showed good to excellent growth at concentrations of 0.001 to 0.064 M. Stronger concentrations were not tested owing to its low solubility. Growth at 0.064 M in most cases exceeded that on the nitrate control.

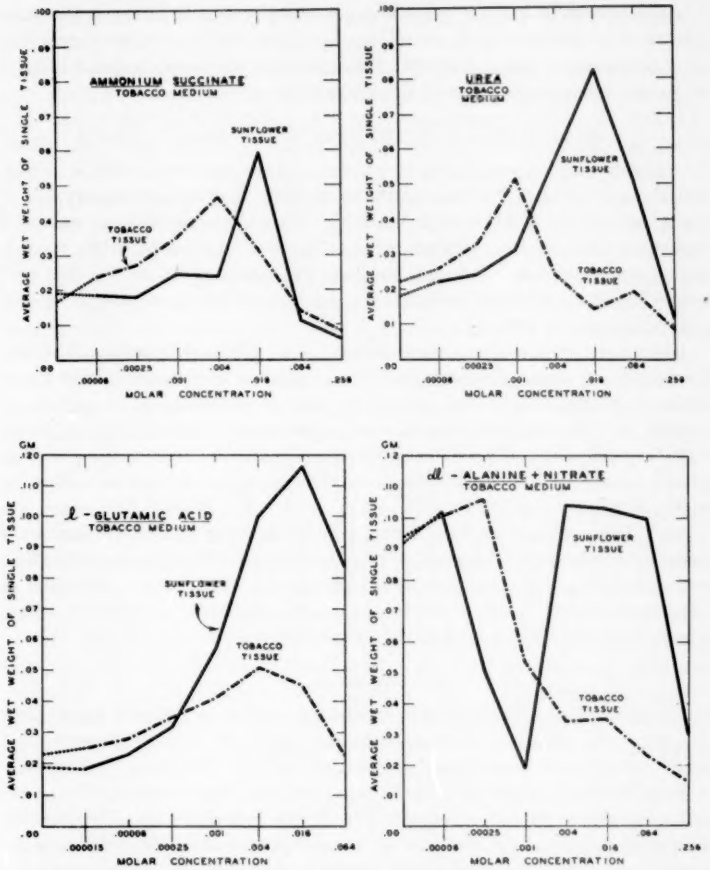
TABLE I

SUMMARY OF THE GROWTH OF TOBACCO AND SUNFLOWER TISSUE *in vitro* ON BASIC MEDIA PLUS VARIOUS SOURCES OF NITROGEN

Source of nitrogen	Minimum number of bottles at each conc.	Amount of growth at molar concentrations of:								
		0.0	.000015	.00006	.00025	.001	.004	.016	.064	.256
A. Tobacco tissue on tobacco medium										
Ammonium succinate	9	±	...	+	+	++	+++	++	±	0
Urea	9	±	...	+	+	++	+	±	±	0
Alanine	8	±	...	±	0	0	±	++++	++++	+
Glutamic acid	9	±	±	±	+	+	++	++	+	...
Leucine	6	±	...	±	0	0	0	0	0	...
Alanine + .004 M. sodium nitrate	8	+++	...	++++	++++	++	+	+	±	0
Leucine + .004 M. sodium nitrate	8	+++	++++	++++	+++	++	++	±	±	...
Alanine + .004 M. urea	8	++	...	+++	++	+	+	++	++	+
B. Sunflower tissue on tobacco medium										
Ammonium succinate	12	±	...	±	±	+	+	+++	0	0
Urea	10	±	...	+	+	++	++++	++++	+++	±
Alanine	11	±	...	+	0	0	+	++	+++	+
Glutamic acid	10	±	±	±	++	+++	++++	++++	++++	...
Leucine	8	±	...	±	+	0	0	0	0	...
Alanine + .004 M. sodium nitrate	7	+++	...	++++	++	±	++++	++++	++++	+
Alanine + .004 M. urea	8	...	++	+++	++	++	++	++	++++	++
C. Sunflower tissue on sunflower medium										
Glutamic acid	7	±	±	++	+++	++++	++++	++++	++++	...
Alanine + .008 M. urea	6	+++	...	++	±	+	++	+++	++++	+

Growth was determined by an approximate comparison of the weights of the tissues with that on the control. It is indicated as follows: 0 = none, ± = a trace, + = ¼ control, ++ = ½ control, +++ = ¾ control, ++++ = equal to, or greater than, control on .008 Molar sodium nitrate.

Companies supplying these materials were as follows: Ammonium succinate from E. H. Sargent & Co., Urea from Merck & Co., DL-alanine, L-glutamic acid, and L-leucine from Pfanstiehl Chemical Co.



FIGS. 1 to 4. Details of representative experiments showing the relative amounts of growth of both tobacco and sunflower tissues on the basic tobacco medium with different amounts, respectively, of several sources of nitrogen. Fig. 1—With ammonium succinate. Fig. 2—With urea. Fig. 3—With glutamic acid. Fig. 4—With alanine plus nitrate.

Tobacco tissue on tobacco medium did not grow as well with L-glutamic acid as did the sunflower tissue (fig. 3). The growth curve was much the same as that for tobacco tissue on DL-alanine, with an optimum at 0.004 to 0.016 M and a definite inhibition of growth above 0.064 M. Growth at optimum concentration was a little over half that on the control. Sunflower tissue on tobacco medium showed good growth at concentrations above 0.001 M with an optimum at 0.064 M, as was the case with DL-alanine on the same medium (fig. 4).

L-leucine alone did not prove a satisfactory source of nitrogen for either sunflower or tobacco tissue on tobacco medium, and all tissues were killed at concentrations above 0.001 M. Occasionally there was a small amount of growth by tobacco tissue at the 0.00006 M concentration.

Substances tested in the presence of sodium nitrate

Sunflower tissue growing on DL-alanine, in the presence of 0.004 M sodium nitrate, showed an inhibition of growth at 0.001 M with a secondary rise in the growth curve at 0.004 M to 0.016 M. This was true both on sunflower and on tobacco medium. Tobacco tissue, however, did not have this secondary growth increase. Tobacco tissue in the presence of DL-alanine, with nitrate constant at 0.004 M, showed a gradual inhibition of growth at concentrations above 0.001 M.

L-leucine, with sodium nitrate constant at 0.004 M, strongly inhibited the growth of tobacco tissue on tobacco medium at concentrations above 0.001 M, though there was some evidence of stimulation of growth at 0.00006 M. This agrees with the results obtained by Riker and Gutsche (**13**) for sunflower tissue on sunflower medium. Periwinkle tissue grown in parallel series on tobacco medium also showed the same type of inhibition with L-leucine.

DL-alanine was also tested in the presence of 0.008 M urea for sunflower tissue on sunflower medium and gave a bimodal growth curve similar to that with DL-alanine in the presence of sodium nitrate. Growth was inhibited at 0.00025 and 0.001 M and there was a marked optimum at 0.064 M, where growth exceeded that on the sodium nitrate control.

Discussion

The growth of tobacco tissue on tobacco medium was fairly constant in all series; the variation between runs in any given series was never very great. Sunflower tissue, grown on tobacco medium, was much more erratic in its behavior. In general, sunflower tissue on tobacco medium gave results comparable with those obtained by Riker and Gutsche (**13**) although the actual weight of the tissues was less and the variation between runs far greater. It can be concluded that sunflower and tobacco tissue differ considerably in their nitrogen requirements and in their reaction to the various nitrogen compounds and that these differences are significant.

The results obtained indicate that tobacco callus tissue and sunflower crown-gall tissue differ considerably in their reactions to different nitrogen sources and it is, therefore, necessary to be careful when making generalizations regarding nitrogen metabolism in specific tissues of higher plants as a whole. The optimum concentrations for the various nitrogen sources tested differ for the two tissues and are generally lower for tobacco tissue than for sunflower. Although the tissues show many differences they resemble each other in that both can utilize urea, DL-alanine, L-glutamic acid, and, to a certain extent, ammonium succinate, as sole sources of nitrogen, and both

tissues show a marked inhibition of growth on L-leucine in the presence of sodium nitrate.

It is interesting to note that alanine, glutamic acid, aspartic acid, asparagine, arginine, and urea are all linked by well known metabolic reactions, and that it was just these organic compounds that RIKER and GUTSCHE (**13**) found to be readily available as sole sources of nitrogen for sunflower tissue.

Glutamic acid, aspartic acid, and alanine may, according to a review by LEONARD and BURRIS (**11**), undergo transamination with oxalacetic, ketoglutaric and pyruvic acids, respectively, so that they are mutually interconvertible, and these transamination reactions are of widespread occurrence in plants. Since glutamic acid can be converted into glutamine (**10**), and aspartic acid into asparagine (**12**), by condensation with ammonia, and since glutamic acid, aspartic acid, and alanine are mutually interconvertible, it is likely that these three amino acids are of special importance in plant metabolism. STEWARD and STREET (**14**) state that in potato the glutamine–glutamic acid system appears especially fitted for the transfer of amino-groups to nitrogen-free metabolites.

In animal tissues, arginine can be broken down to give either urea or ammonia, and WOMACK and ROSE (**16**) found that arginine, proline, ornithine, and glutamic acid are all mutually interconvertible. DAMODARAN *et al.* (**3**) showed that in germinating seedlings the disappearance of arginine is associated with an increase in the amount of asparagine and glutamine present, so, presumably, there is a relationship between these substances.

The results obtained for the growth of sunflower tissue with alanine in the presence of sodium nitrate suggest that these substances inhibit the utilization of nitrate at the 0.001 M level. This inhibition also appeared when 0.008 M urea was used instead of nitrate. The phenomenon seems related to changes in the amino-acid balance in the tissues, but its explanation must wait until more data have been obtained.

Since tobacco and sunflower tissue both utilized alanine and glutamic acid as sources of nitrogen, one would expect them to give similar growth curves with alanine in the presence of sodium nitrate, but this was not the case. The absence of any distinct inhibition and secondary rise in the growth curve in the case of tobacco tissue, may be due to the fact that the optimum concentration for tobacco growing on DL-alanine is lower than that for sunflower tissue, and approaches too closely the inhibiting concentration.

It seems reasonable to suppose that the differences in the reactions of the various plant tissues to different nitrogen sources are an expression of variations in the amino-acid balance necessary for active protein synthesis in the plant cell.

Summary

The growth of tobacco tissue *in vitro* on synthetic media was studied and compared with that of sunflower tissue on the same medium. Six nitro-

gen compounds were tested and the results obtained compared with those of RIKER and GUTSCHE (**13**). A wide range of concentrations was used. The growth of tobacco tissue was less than that of sunflower in every case, and the optimum concentration for each substance was lower for the tobacco tissue.

Ammonium succinate alone did not support good growth of either species. The growth at optimum concentration was about two-thirds that on the nitrate control in each case.

Urea was an excellent source of nitrogen for sunflower tissue, allowing better growth than the nitrate control; but with tobacco tissue, growth was only half that on the control, and the optimum concentration was only 0.001 M against 0.016 M for sunflower.

DL-alanine was a good source of nitrogen for both tissues. Growth was almost as good as on the control. The optimum concentration for sunflower was 0.064 M, for tobacco, 0.016 M. L-glutamic acid supported excellent growth of sunflower tissue at 0.016 M, but for tobacco the optimum concentration was only 0.004 M, and growth was only half that on the control.

L-leucine alone did not support growth of either tissue, and it inhibited growth in the presence of sodium nitrate. In tobacco tissue the inhibition was not as marked as in sunflower and there was some increased growth at the 0.00006 M level.

Sunflower tissue on DL-alanine in the presence of sodium nitrate gave a bimodal growth curve, with complete inhibition at 0.001 M. The same type of growth curve was obtained with alanine in the presence of 0.008 M urea. Tobacco with alanine in the presence of 0.004 M sodium nitrate showed no distinct inhibition and secondary rise in the growth curve.

Plant tissues apparently vary greatly in their response to different sources of nitrogen. These differences may be associated with variations in the critical amino-acid balances of the tissues.

This work received support from the American Cancer Society, the Donner Foundation, and from the University of Wisconsin Research Committee with funds provided by the Wisconsin Alumni Research Foundation. Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

The writers are indebted to Professors R. Burris and F. K. Skoog for valuable suggestions received and to Eugene Herrling for preparation of figures.

DEPARTMENT OF PLANT PATHOLOGY
UNIVERSITY OF WISCONSIN
MADISON, WISCONSIN

LITERATURE CITED

1. BÜRSTROM, HANS. The nitrate nutrition of plants. A general survey of the occurrence and assimilation of nitrate. Ann. Royal Agric. Coll. Sweden **13**: 1–86. 1945.

2. CHIBNALL, A. C. Protein metabolism in the plant. 306 p. Yale Univ. Press, New Haven. 1939.
3. DAMODARAN, M., RAMASWAMY, R., VENKATESAN, T. R., MAHADEVAN, S., and RAMDAS, K. Amide synthesis in plants. II. Amino-acid changes in germinating seedlings. Proc. Indian Acad. Sci. Sec. B. **23**(2): 86–99. 1946.
4. GAUTHERET, R. J. La culture des tissus. 203 p. Lagny-sur-Marne, Paris. 1945.
5. HILDEBRANDT, A. C. and RIKER, A. J. Influence of some growth-regulating substances on sunflower and tobacco tissue *in vitro*. Amer. Jour. Bot. **34**: 421–427. 1947.
6. HILDEBRANDT, A. C. and RIKER, A. J. The influence of various carbon compounds on the growth of marigold, Paris-daisy, periwinkle, sunflower and tobacco tissue *in vitro*. Amer. Jour. Bot. **36**: 74–85. 1949.
7. HILDEBRANDT, A. C., RIKER, A. J., and DUGGAR, B. M. Growth *in vitro* of excised tobacco and sunflower tissue with different temperatures, hydrogen-ion concentrations, and amounts of sugar. Amer. Jour. Bot. **32**: 357–361. 1945.
8. HILDEBRANDT, A. C., RIKER, A. J., and DUGGAR, B. M. Influence of crown-gall bacterial products, crown-gall tissue extracts, and yeast extract on growth *in vitro* of excised tobacco and sunflower tissue. Cancer Res. **6**: 368–377. 1946a.
9. HILDEBRANDT, A. C., RIKER, A. J., and DUGGAR, B. M. The influence of the composition of the medium on the growth *in vitro* of excised tobacco and sunflower tissue cultures. Amer. Jour. Bot. **33**: 591–597. 1946b.
10. KREBS, H. A. Metabolism of amino-acids. IV. The synthesis of glutamine from glutamic acid and ammonia, and the enzymic hydrolysis of glutamine in animal tissues. Biochem. Jour. **29**: 1951–1969. 1935.
11. LEONARD, M. J. K. and BURRIS, R. H. A survey of transaminases in plants. Jour. Biol. Chem. **170**: 701–709. 1947.
12. MOTHES, K. Zur biosynthese der säureamide asparagin und glutamin. Planta **30**: 726–756. 1940.
13. RIKER, A. J. and GUTSCHE, ALICE E. The growth of sunflower tissue *in vitro* on synthetic media with various organic and inorganic sources of nitrogen. Amer. Jour. Bot. **35**: 227–238. 1948.
14. STEWARD, F. C. and STREET, H. E. The nitrogenous constituents of plants. Ann. Rev. Biochem. **16**: 471–502. 1947.
15. WHITE, P. R. A handbook of plant tissue culture. 277 p. The J. Cattell Press, Lancaster, Pa. 1943.
16. WOMACK, M. and ROSE, W. C. The role of proline, hydroxyproline and glutamic acid in growth. Jour. Biol. Chem. **171**: 37–50. 1947.

RESPIRATION STUDIES ON CHLORELLA. II. INFLUENCE OF VARIOUS ORGANIC ACIDS ON GAS EXCHANGE

DÉSIRÉ M. ENY[1]

(WITH TWELVE FIGURES)

Received July 25, 1950

Introduction

In an attempt to elucidate the respiratory mechanism of the green alga, chlorella, several of the organic acids which are known to be intermediates in the aerobic path of carbohydrate metabolism in most animals and bacteria were supplied to chlorella cells. In a previous paper (**9**), the results of growth experiments were reported and there was some evidence of utilization of the acid intermediates for growth in the dark by chlorella.

Since the organic acids supplied are assumed to enter the path of metabolism through the respiratory mechanism, the effects of the organic acids on the respiratory gas exchange (*i.e.*, oxygen uptake and carbon dioxide evolution) were investigated. The results of this investigation are reported here.

Materials and methods

The effect of the possible acid intermediates of the aerobic cycle of carbohydrate metabolism on the respiration of chlorella was studied by manometric measurements with the Warburg apparatus. All determinations were made in the dark. For this purpose, the instrument was placed in a blackened chamber equipped with an air-circulating fan to avoid rises in air and bath temperatures. The rate of shaking was adjusted to 200 two-centimeter strokes per minute. The flasks used were of the 2-side-arm, center-well, and venting-plug type. The bath temperature was regulated at 28° C. The vessel constants were calculated by the UMBREIT, BURRIS, and STAUFFER method (**17**).

The measurements of oxygen and of carbon dioxide were determined by using a pair of flasks, one of which contained KOH in the center cup to absorb the CO_2. The KOH was replaced by water in the other flask. Both flasks also contained an accordion-pleated filter paper, 2×2 centimeters square, to increase the CO_2 absorbing surface in the first flask and to equalize the volume of the gas phase in the other.

Nutrient solutions and cell suspensions added to the flasks were prepared similarly to those used for the growth experiments (**9**). The solution referred to as plain nutrient solution is composed of 1.25 gm. KNO_3, 2.46 gm. $MgSO_4 \cdot 7\,H_2O$, 1.22 gm. KH_2PO_4, 0.01 gm. $FeCl_3$, 1 ml. of Haas and Reed A–Z solution, and 1,000 ml. H_2O. The initial pH of the solution was regulated with a few drops of normal HCl to 5.4–5.6 which was found to be

[1] Present address: Firestone Plantations, Harbel, Liberia, W. Africa.

favorable for the growth of chlorella. This gives a system fairly well buffered. The buffer properties of this solution are further increased by the addition of organic acids. The cells were starved for 30 hours in the dark at 28° C before use but no attempt was made to maintain aseptic conditions in the Warburg flasks. Due to the desirability of using cells which were kept only a short period of time in the refrigerator (less than 10 days), it was not possible to use the same concentration of cells for all the experiments. Not enough cells were available from one stock suspension.

Several experiments were conducted with cell extracts replacing the cell suspensions. The extracts were prepared in several ways—by grinding the chlorella with very fine carborundum in a mortar, by using powdered glass and sand as abrasives, and also by grinding in a tissue homogenizer. The ground cells were stirred with two to three milliliters of triple-distilled water. After centrifuging, the supernatant liquid was used as the cell extract.

The system in each flask was composed of:

a) the cell suspension (1 milliliter) which was placed into the main compartment of the flask together with the necessary volume of water and nutrient solution to total five milliliters of liquid, and

b) the organic acids which were placed in the side arms and tipped into the main compartment after equilibrium had been reached.

The thermobarometer flask was set up with the same material as the other containers except for the cell suspension which was replaced by an equal volume of water. The concentrations of the cells and of the organic acids were varied to obtain results which would show most clearly the effect on respiration.

The organic acids used were the same as those used in the growth experiments. α-ketoglutarate, however, was added to the list as it was available in small quantity.

Results

Before proceeding with the experiments showing the effect of organic acids on respiration, several preliminary determinations were made to compare the value of various inorganic media. With a view toward selecting the solution which would give best results for respiration studies, the oxygen uptake and carbon dioxide evolution were measured for several systems:

a) Krebs-Ringer phosphate solution + chlorella,
b) Krebs-Ringer bicarbonate solution + chlorella,
c) phosphate buffer, pH 5.6, + chlorella,
d) the nutrient solution with various inorganic salts added + chlorella,
e) various concentrations of the plain nutrient solution + chlorella.

The first three media gave poor results in that the curves of the O_2 uptake and the CO_2 evolution were lower than those with plain nutrient solution. Varying the concentration of the nutrient solution led to the selection of $\frac{1}{3}$ normal concentration as the one giving the most desirable curve

features. Inorganic salts were added to the ⅓ nutrient solution in the hope that they would produce higher respiratory rates. The curves obtained were not as regular and the respiratory quotient [2] varied more than that with the plain nutrient solution. The effect of these inorganic salts is shown in figure 1. The cell concentration for this figure was 1.4×10^8 per flask.

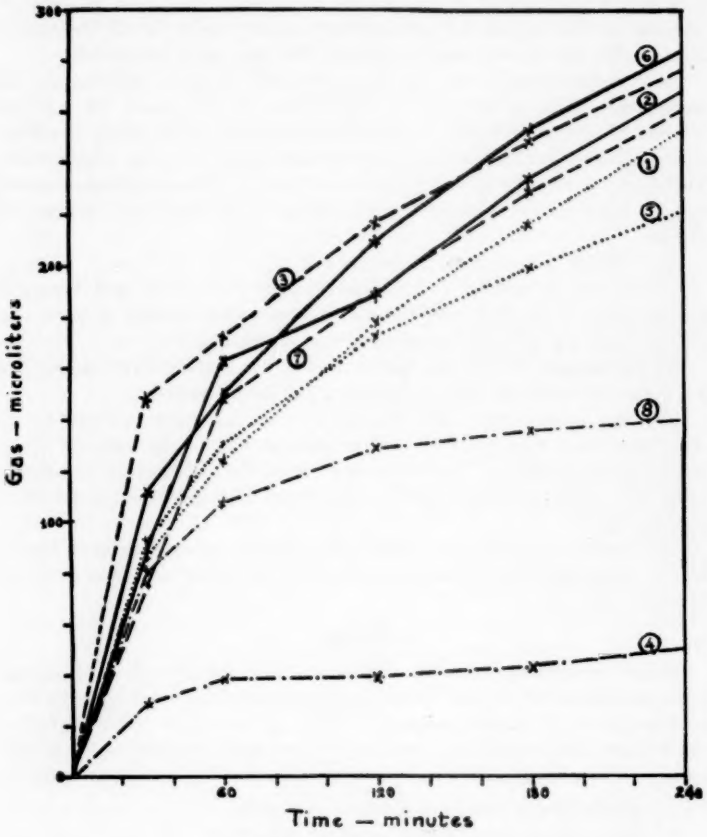


FIG. 1. Inorganic salts.

It can be seen that the QO_2 (the microliters of oxygen uptake for 10^9 cells per hour) and QCO_2 (the microliters of carbon dioxide evolution for 10^9 cells per hour) were highest when 0.006 M monosodium phosphate (curves

[2] The respiratory quotient (R.Q.) throughout does not take into account CO_2 fixation.

2 and 6) or 0.006 M monopotassium phosphate (curves 3 and 7) were added to the nutrient solution. The QO_2 and QCO_2, obtained with 0.006 M KCl, were lower than for the nutrient solution containing no added salts. The addition of 0.005 M $Ca(NO_3)_2$ gave curves very similar to those obtained with KCl. Because of the variations of R.Q. produced upon the addition of phosphate, the plain nutrient solution was used without the addition of inorganic salts in all the following experiments.

The data obtained for all the respiratory experiments are shown in table I. Values have been reported for the periods of time of 120, 130 and 240 minutes only.

Effect of glucose

Before studying the effect of the organic acids on the respiration of chlorella in the dark, it was desirable to have some basis of comparison. Respiration curves were obtained with starved cells placed in nutrient solutions containing several concentrations of glucose and also with no glucose added (as control). It was found that little variation occurred even when the concentrations ranged from 0.005 to 0.1 M, probably because even a very little glucose is sufficient to give a good respiration rate. However, as a glucose concentration of 0.08 M gave the best growth in the experiments reported in the previous section, the respiration data are shown for that particular concentration (fig. 2). The number of cells was 4.3×10^7 per flask.

The QO_2 and QCO_2 of the cells grown in glucose (curves 1 and 3) are considerably higher than those of the control (starved cells—curves 2 and 4). Although the respiration of the starved cells is appreciable, it would appear that their respiratory mechanism is slowed but not drastically affected. Even when starvation was more severe (up to three days in the dark at about 30° C), the O_2 curve was close to the same level. The CO_2 curve, however, dropped more rapidly. In the experiment reported in figure 2, it seems that the cells were not starved sufficiently. In spite of the fact that they were left the fixed length of time in the dark, the R.Q. was about unity. (The cells used in the following respiratory experiments, however, gave R.Q.'s below unity as shown by the low level of the CO_2 curves. These cells, from stock suspensions prepared at different times, were apparently more deprived of carbohydrates. In such case, the respiration rate should be lower and the relative amount of CO_2 fixation higher.)

An interesting characteristic of the cells in glucose is that they maintained a constant respiration rate for a longer period of time than the cells in plain nutrient solution. The starved cells were more readily affected by a decrease in gas pressure in the flasks. If air was admitted into the flasks by opening the manometers, the same-shaped curves were again obtained, which shows that the drop in respiration of starved cells is related to the decrease of gas pressure in the flasks.

Respiration curves of starved cells from the same stock suspension,

TABLE I

EFFECT OF VARIOUS COMPOUNDS ON THE RESPIRATION OF CHLORELLA

Compound added and molar concentration	Figure	Curve	Microliters of O_2 Uptake: In each experiment Time in Minutes 120	180	240	For 10^9 cells per hour	Curve	Microliters of CO_2 Evolved: In each experiment Time in Minutes 120	180	240	For 10^9 cells per hour
0.00		1	178.0	215.0	254.4	450	5	171.0	199.0	221.3	400
0.006 $NaH_2PO_4 \cdot H_2O$		2	188.0	237.0	266.8	476	6	209.0	253.0	284.0	507
0.006 KH_2PO_4		3	185.4	228.0	260.0	464	7	216.0	248.0	275.4	491
0.006 KCl	1	4	38.0	43.0	49.7	89	8	128.0	134.0	140.5	253
0.08 Glucose		1	117.2	171.0	224.2	1,307	3	124.4	177.5	230.6	1,340
0.00	2	2	52.6	70.0	78.3	456	4	63.1	84.2	97.7	568
0.30 Lactate		1	23.5	38.2	52.1	930	4	31.5	57.9	84.6	1,510
0.05 "		2	12.4	21.7	39.2	700	5	26.1	40.6	58.1	1,038
0.00	3	3	18.3	26.7	31.4	561	6	9.8	16.6	19.5	348
0.20 Pyruvate		1	164.0	251.0	338.0	704	4	40.0	59.0	80.0	166
0.05 "		2	263.4	372.6	490.2	1,020	5	133.0	207.0	263.0	547
0.00	4	3	160.0	204.0	226.0	470	6	74.0	133.4	158.2	329
0.15 Acetate		1	281.6	403.3	522.7	1,537	4	146.1	223.7	300.4	884
0.05 "		2	182.4	278.3	379.4	1,115	5	126.8	177.2	238.2	701
0.00	5	3	146.8	171.7	191.3	563	6	114.5	122.1	146.8	432
0.15 Aconitate		1	55.6	66.7	69.3	1,237	4	32.2	37.4	39.5	705
0.03 "		2	39.2	42.8	45.5	812	5	15.5	25.7	30.6	546
0.00	6	3	24.2	27.6	29.6	529	6	21.6	27.0	25.7	459
0.30 Citrate		1	24.2	31.0	34.0	121	5	19.0	11.0	11.5	41
0.10 "		2	52.6	71.0	88.1	315	6	18.2	23.0	25.6	91
0.02 "		3	82.3	117.6	143.5	513	7	38.1	50.0	58.1	208
0.00	7	4	90.2	124.0	158.0	564	8	71.0	105.0	138.0	492

TABLE I—(*Continued*)

Compound added and molar concentration	Figure	Curve	Microliters of O_2 Uptake				Curve	Microliters of CO_2 Evolved			
			In each experiment Time in Minutes			For 10^9 cells per hour		In each experiment Time in Minutes			For 10^9 cells per hour
			120	180	240			120	180	240	
0.06 α-keto glutarate		1	108.0	132.0	142.0	825	4	56.1	90.2	112.0	651
0.02 " "		2	90.4	101.2	108.0	628	5	34.0	54.4	67.2	391
0.00	8	3	63.6	81.7	90.3	525	6	48.3	65.0	78.2	455
0.15 Succinate		1	25.6	41.3	54.7	595	4	22.8	37.3	51.4	559
0.03 "		2	27.8	43.5	58.6	637	5	18.3	30.2	42.9	466
0.00	9	3	21.3	34.7	46.4	504	6	16.8	28.1	38.6	420
0.15 Fumarate		1	38.8	49.5	66.7	1,191	4	10.7	17.8	38.0	678
0.03 "		2	26.1	33.5	42.4	757	5	18.3	24.0	31.4	560
0.00	10	3	23.1	27.8	30.0	536	6	14.6	19.0	22.1	395
0.15 Malate		1	26.3	38.0	50.3	898	4	24.6	35.7	47.2	843
0.03 "		2	9.2	13.8	19.6	350	5	7.8	10.9	15.7	280
0.00	11	3	16.6	23.8	28.4	507	6	8.1	15.8	20.3	363
0.15 Butyrate		1	24.1	29.3	34.2	855	4	17.8	21.6	25.8	645
0.05 "		2	13.7	18.8	23.2	580	5	12.6	17.3	21.4	535
0.00	12	3	13.0	18.2	22.0	550	6	7.8	10.7	14.3	358
0.15 Propionate		7	14.1	20.0	25.8	645	9	5.6	7.8	10.2	255
0.05 "		8	11.3	17.0	20.4	510	10	2.6	4.4	6.1	153
0.00	12	3	13.0	18.2	22.0	550	6	7.8	10.7	14.3	358

grown in the same media but at different times, were not identical. To determine the effect of the organic acids on respiration, it was then necessary to conduct, with each experiment, a determination of the cell respiration in plain nutrient solution. These curves were used as controls.

Many concentrations of acids were used, but only the most significant results have been selected for the tables.

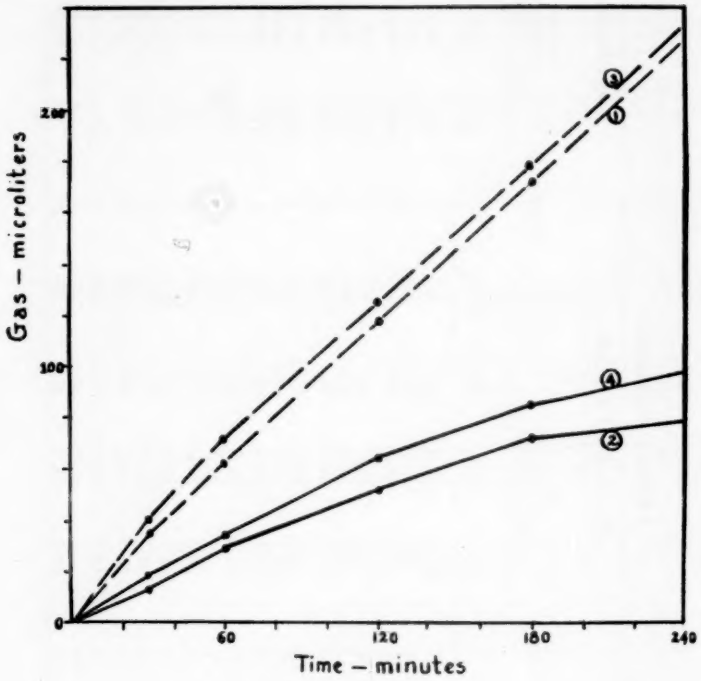


FIG. 2. Glucose.

EFFECT OF ORGANIC ACIDS

SODIUM LACTATE.—The effect of lactate on respiration is shown in figure 3. The cell concentration for this figure was 1.4×10^7 per flask. The highest respiration rate was produced at 0.3 M concentration. Above this, the respiration began to decrease rapidly and at 0.6 M, it was below the level of the control. Despite the fact that as low as 0.005 M indicated a slight amount of growth, an increase of respiration over the control became noticeable only when 0.01 M lactate was furnished. At 0.05 M, the increase was already considerable, as shown by the QO_2 of 700 microliters.

A notable feature of the respiration with lactate is that it is the only acid

studied which gave an apparent R.Q. higher than unity (*i.e.*, R.Q. at 240′ for 0.05 M = $\frac{1038}{700}$ = 1.48). Such a high R.Q. is not extraordinary. GAFFRON (10) found that the R.Q. of several species of algae (*Chlorella pyrenoidosa* and others) was abnormally high (1.2 to 2.0) when the algae were cultivated in various concentrations of glucose.

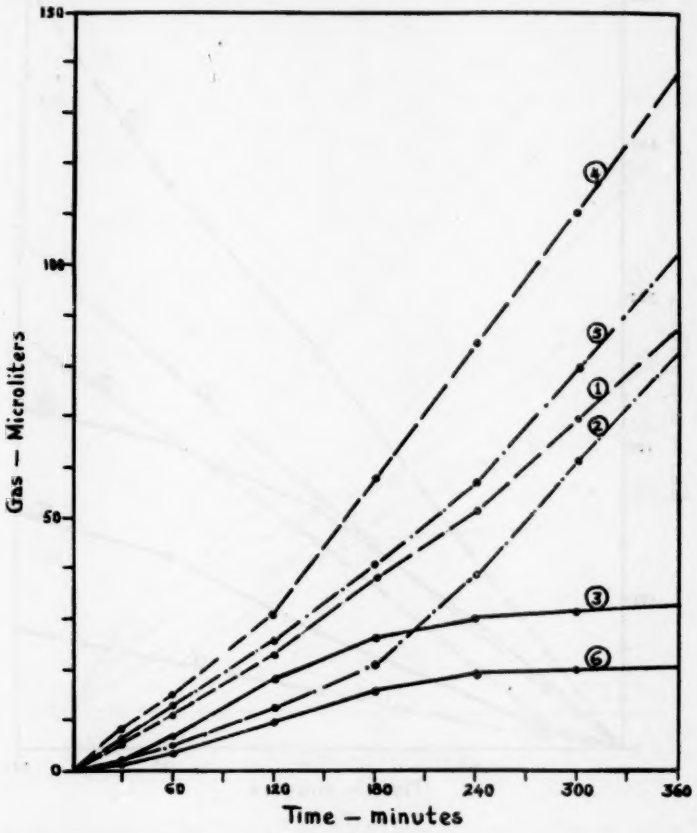


FIG. 3. Lactate.

The high R.Q. obtained with lactate may indicate that carbohydrates are being formed more rapidly with lactate than with the other acids, by the reversal of a process similar to glycolysis, and/or that in this case, there is little dark CO_2 fixation taking place.

SODIUM PYRUVATE.—The respiration of chlorella, when pyruvate was added to the nutrient solution, is shown in figure 4. The cell suspension

had a concentration of 1.2×10^8 per flask. It can be seen that the highest QO_2 was reached when the acid concentration was 0.05 M. At this concentration, the QCO_2 was higher than that of the control but it remained at a fairly low level, which denotes either an extremely low R.Q. or a large amount of CO_2 fixation, or both.

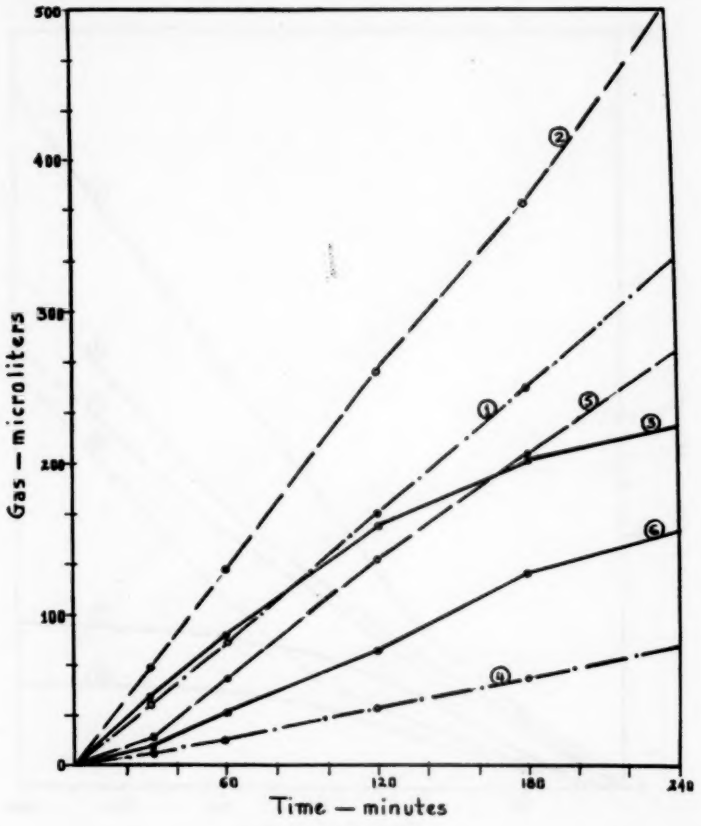


FIG. 4. Pyruvate.

Between the concentrations of 0.03 M and 0.08 M, the respiration did not change appreciably. However, below and above these concentrations, the respiration level fell. Curves 1 and 4 show this effect, obtained with 0.2 M pyruvate. The O_2 uptake remained slightly higher than for the control with as low as 0.001 M pyruvate.

Data on the respiration rate up to four hours are shown. Little significance can be obtained after this time. Between six and eight hours, the rates of respiration decreased. These effects are due mainly to decreased

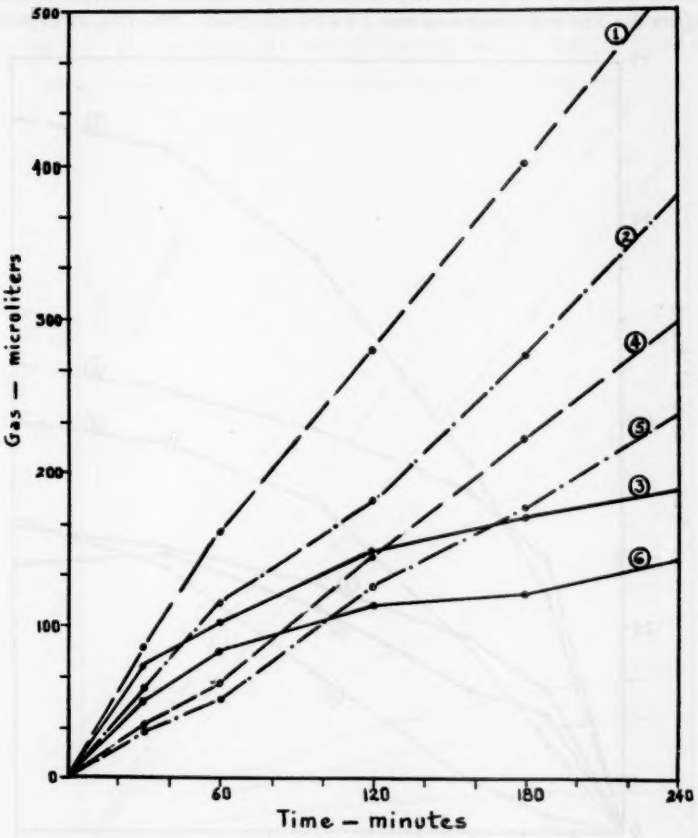


FIG. 5. Acetate.

oxygen pressure in the flasks. After 15 hours, the respiration with 0.05 M pyruvate was, however, still above that of the control.

SODIUM ACETATE.—Even at a very low concentration such as 0.005 M, acetic acid produced an appreciable amount of respiration over that of the control. As the concentration increased up to 0.6 M, respiration also increased. Above that concentration, within a 30-minute period after the acid was added, the curve of the O_2 uptake climbed rapidly and then fell

below the control. Figure 5 gives the results obtained for acetic acid concentrations of 0.15 M and 0.05 M with a suspension containing 8.5×10^7 cells. In both cases, the QO_2 was above the control.

SODIUM ACONITATE.—The respiration data for aconitic acid are shown in figure 6. The cell suspension was 1.4×10^7 per flask. The QO_2 and QCO_2

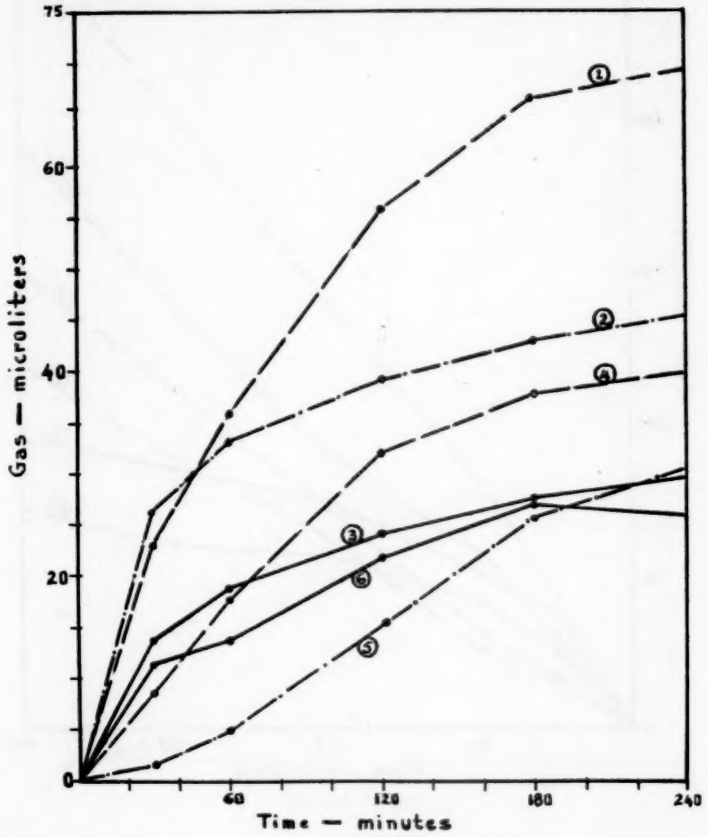


FIG. 6. Cis-Aconitate.

are higher with aconitic acid than with the control. It can also be seen that the QCO_2 is lower than the QO_2 which indicates that the R.Q. is smaller than 1 and, consequently, there must be some CO_2 fixation. This is in agreement with the growth results obtained. The rate of respiration increased with the concentration of aconitate (up to about 0.6 M), the best growth occurring with an 0.04 M concentration. A possible explanation is that after periods varying from several hours to several days, the respira-

tion rate of chlorella in high aconitate concentrations drops. This type of behavior was observed also in the case of acetate.

SODIUM CITRATE.—With all the concentrations of citrate used, the respiration curve for chlorella did not reach that of the control. The results obtained for 7×10^7 cells per flask with various levels of citrate are shown in figure 7. The maximum QO_2 was obtained for 0.02 M, when the O_2 curve

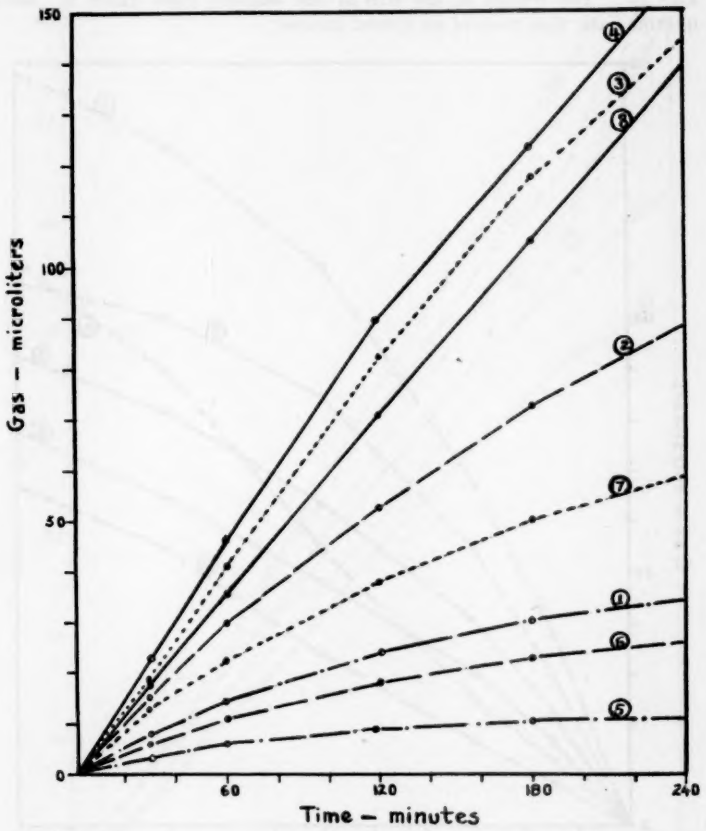


FIG. 7. Citrate.

closely approached that of the control, but remained lower nevertheless. The QCO_2 was definitely below the control. These conditions were much the same when citrate was varied from 0.01 M to 0.04 M. Above these limits the respiration rate fell more rapidly (curves 1 and 4).

With 0.02 M citrate, a small amount of growth was obtained, yet the respiration curves indicate some inhibition but to a lesser degree than with

other concentrations. It may be that a sort of adaptation takes place in the chlorella after some time and results in a change in the respiratory rate sufficient to produce assimilation of citrate and growth.

SODIUM α-KETOGLUTARATE.—Due to the small amount of α-ketoglutarate available, the respiration of chlorella was measured with only three concentrations—0.002 M, 0.04 M and 0.06 M. The number of cells per flask was 4.3×10^7. The results of the 0.04 M are omitted from figure 8. Being intermediate, they were of no special interest.

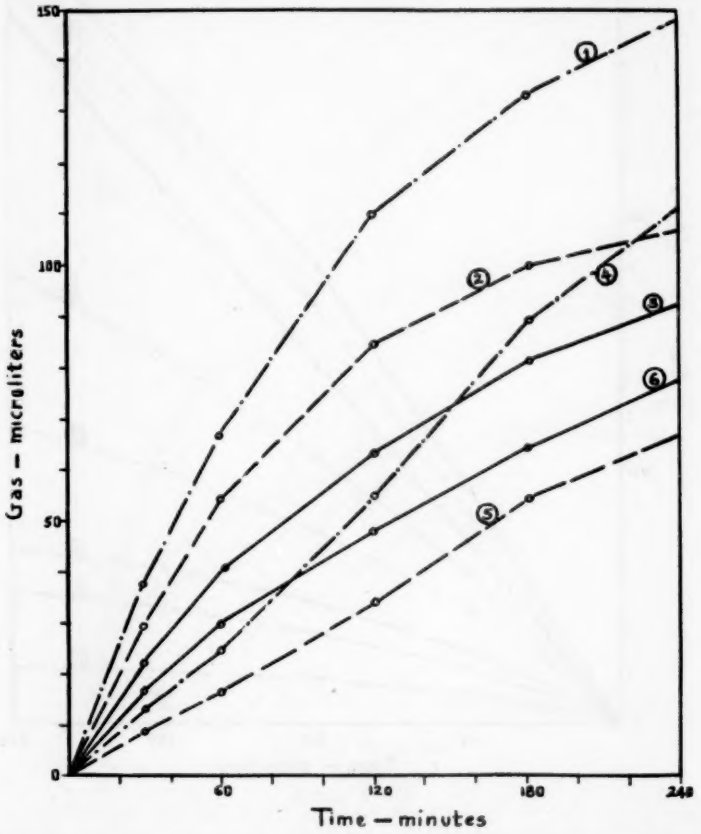


FIG. 8. α-Ketoglutarate.

It may be concluded that α-ketoglutarate was respired as the QO_2 in both cases is higher than that of the control. Oxidative assimilation is indicated by the low R.Q.

The comparison of these curves with those of the other acids signifies that α-ketoglutarate probably is a source of organic matter for chlorella growth. These respiration data, however, cannot alone be taken as final criteria for such a conclusion.

SODIUM SUCCINATE.—Succinate did not produce much rise in respiration generally. As shown in figure 9, the highest rate of respiration was ob-

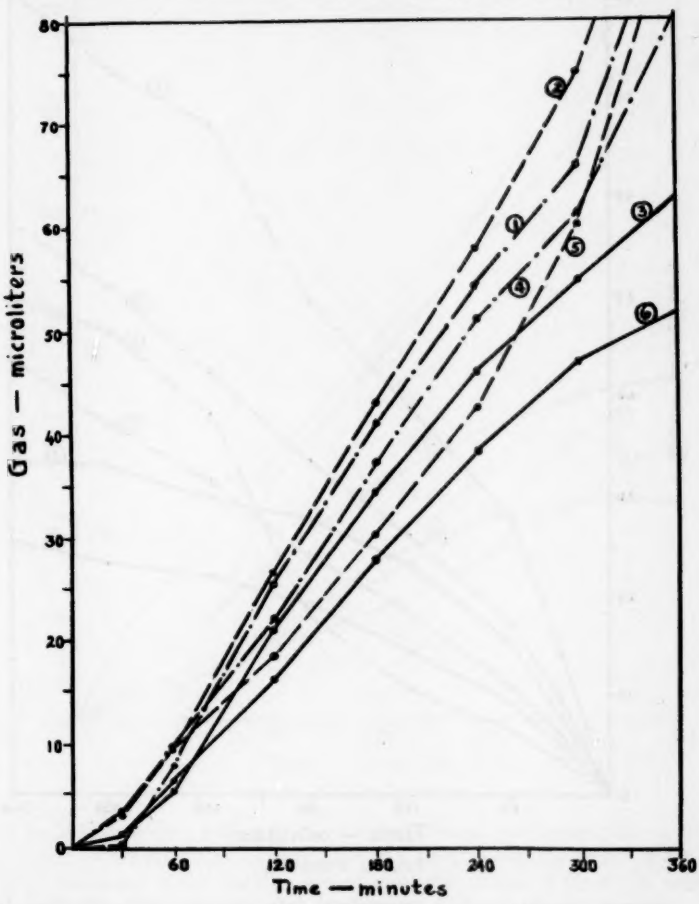


FIG. 9. Succinate.

tained for an 0.03 M concentration with 2.3×10^7 cells per flask (curves 2 and 5). In that instance, the QO_2 was 637 microliters as against 504 microliters for the control.

Up to 0.15 M, cells in succinate absorbed more oxygen than the control, but when 0.3 M succinate was used, the respiratory rate fell below the control level.

It can be seen that with succinate the O_2 uptake is higher in all cases than the CO_2 evolution, giving a R.Q. lower than 1.0.

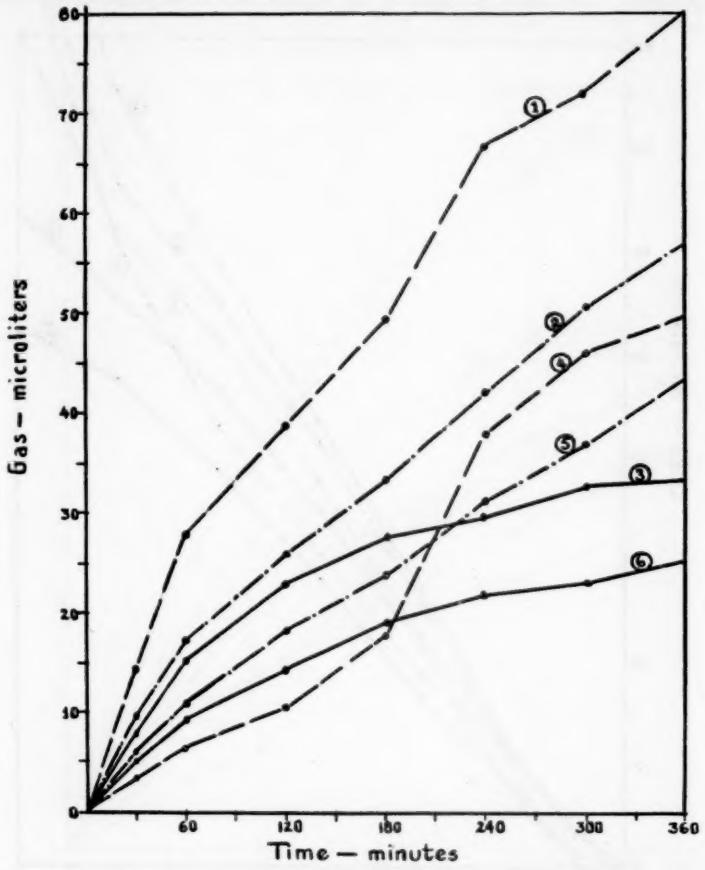


FIG. 10. Fumarate.

SODIUM FUMARATE.—The respiratory data obtained with fumarate for 1.4×10^7 cells per flask are shown in figure 10. These results are generally similar to those obtained for aconitate, the only noticeable difference being that the curves for fumarate are consistent in trend, but more irregular in shape.

SODIUM MALATE.—The data on the respiration of chlorella with malate are presented in figure 11. For the first four hours, the QO_2 of the cells

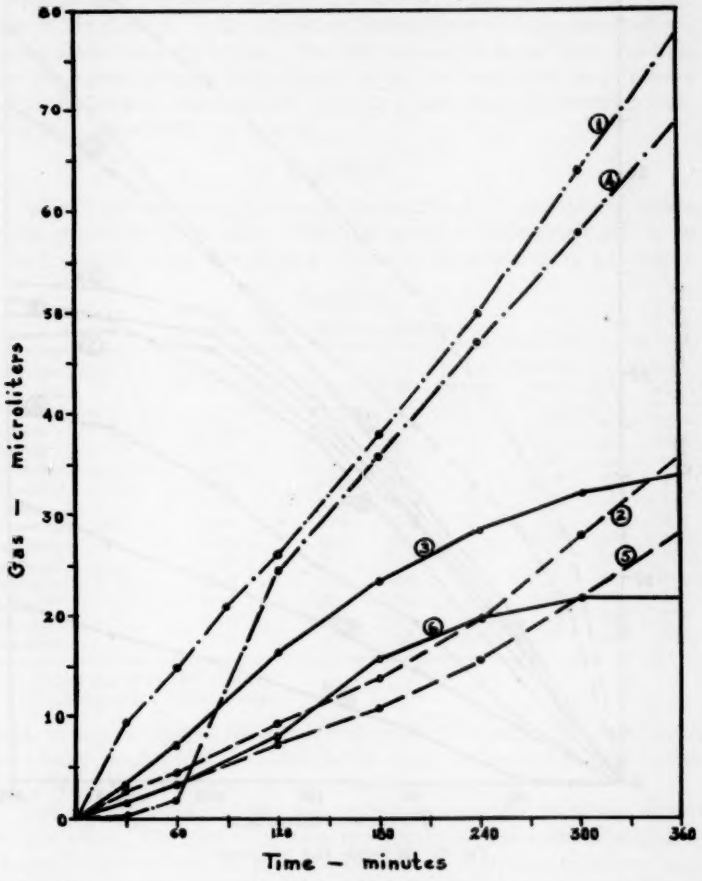


FIG. 11. Malate.

(1.4×10^7 per flask), with an 0.03 M concentration, was below that of the control. After six hours, however, the QO_2 became slightly higher and the trend continued in this manner for an indefinite period of time. In other respects these data are comparable to those for aconitate and fumarate.

SODIUM BUTYRATE.—The effect of butyrate on respiration was hardly noticeable until a concentration of about 0.02 M was supplied. For 0.05 M, the effect was negligible at first, but, after six hours and over, respiration became appreciably higher than that of the control. Figure 12 shows the

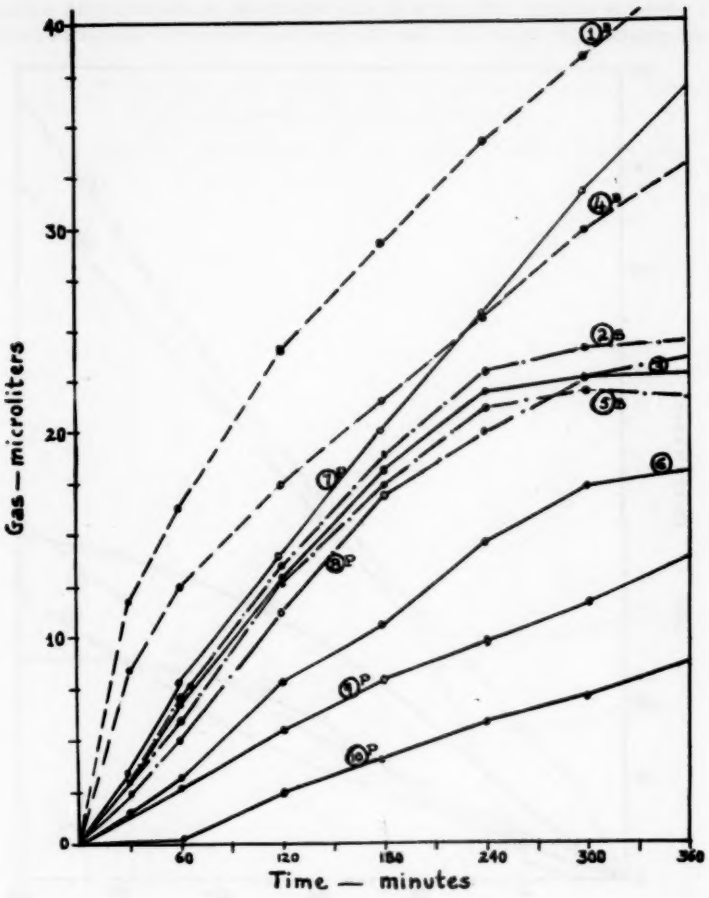


FIG. 12. Butyrate and propionate.

curves obtained for 1×10^7 cells per flask. At 0.15 M, the respiration rate was higher than at 0.05 M, as can be seen by the QO_2 and by the level of curve 1, but the growth was poorer. It is possible that, similar to lactate, the higher respiration rate does not last as long as with lower concentrations of acids or that some toxicity, due to physical or chemical causes, enters into being. It may be noted that the R.Q. is very close to unity, $(\frac{535}{580} = 0.92)$, which may indicate a low amount of CO_2 fixation and may explain the low ratio of growth obtained in the previous experiments.

SODIUM PROPIONATE.—The respiratory level obtained with propionate was generally low. The curves obtained with 1.4×10^7 cells per flask are

shown in figure 12. As much as 0.15 M propionate was necessary to raise the QO_2 to 645; at lower concentrations the oxygen uptake decreased. During the first five hours, the curves obtained for 0.05 M were lower than those of the control. After about six hours, however, they rose and remained above the control level. The R.Q. calculated at six hours was 0.38. This low value indicates that, in spite of the low respiration level, growth and the building of organic matter could have been obtained through a relatively large amount of CO_2 fixation.

Discussion

Table II gives a general picture of the results of the organic acid effects on the respiration of chlorella. The CO_2 values of the controls are shown in each case for better comparison. It can be seen that there was only a

TABLE II

SUMMARY OF RESPIRATION DATA

Organic acid salt	Concentration	Q_{O_2}	Q_{CO_2}	Order number	Growth ratio	Control Q_{O_2}	Control Q_{CO_2}
Lactate	0.05	700	1,038	6	4.10	470	329
Pyruvate	0.05	1,020	547	2	4.0	561	348
Acetate	0.05	1,115	701	1	3.97	563	432
Aconitate	0.03	812	546	4	2.97	529	459
Citrate	0.02	513	208	9	1.65	564	492
α-keto glutarate ..	0.06	825	651	3	....	525	455
Succinate	0.03	637	466	7	1.67	504	420
Fumarate	0.03	757	560	5	2.56	536	395
Malate*	0.03	350	280	11	2.23	507	363
Malate**	0.03	428	334	11	2.23	410	259
Butyrate	0.05	580	535	8	2.05	550	358
Propionate	0.05	510	153	10	1.57	550	358

*Calculated at 240 minutes.
**Calculated at 360 minutes.

small change in the QO_2 for the starved cells. The QCO_2, however, varied on a wider range which is related to the degree of starvation of the cells, the QCO_2 being smaller for the more starved cells.

The oxygen uptake of the cells varied considerably with the several organic acids. The relative order of this uptake is to be found in column 5, acetate being first with the highest value. It is interesting to observe that the order does not correlate exactly with the relative order obtained for the growth experiments. A good relationship exists nevertheless, as pyruvate, lactate, acetate and aconitate gave the highest growth and citrate, succinate and propionate, the lowest.

Beyond a certain concentration level, all of the acids studied produced a decline in respiration below that of the controls, due probably to physical or chemical toxicity.

For most of the acids (lactate, acetate, aconitate, fumarate, malate, butyrate and propionate), the oxygen uptake was greater with a concentra-

tion higher than that which gave the best growth results. It is possible that after a certain length of time, shorter than the growth period, the respiration rate falls, as in the case of acetate, or that, with a high respiration rate, the amount of CO_2 evolved is large and the relative CO_2 fixation becomes insufficient to make up for the loss of carbon and to build organic materials for growth.

It was noted that the R.Q. for all of the acids except lactate was below unity. This low R.Q. may be due to three possible causes:

1) The acids were assimilated and a large quantity of CO_2 was fixed for the possible building of carbohydrates and the accumulation of organic matter.

2) Respiration was due to the oxidation of proteins and fats as primary products. This is a possibility which may exist conjointly with the first mentioned.

3) Even without any CO_2 fixation, the amount of acid breakdown is insufficient to give a high CO_2 evolution.

The positive results obtained in the growth experiments definitely favor the oxidative assimilation and CO_2 fixation theory.

The carboxylation of 3- and 4-carbon acids and the fixation of carbon dioxide in the dark has been reported in various plant tissues. Wolf (**21**) showed that pyruvic acid added to leaf strips caused a marked decrease in the CO_2 evolution. Ruben, Hassid, and Kamen (**16**), using radioactive CO_2, observed the fixation of carbon dioxide in the dark in barley leaves and in chlorella suspensions.

Barker (**3**), Clifton (**7**), Doudoroff (**8**), and Anderson (**2**) have demonstrated the generality of an oxidative assimilation of carbon in microorganisms. It is illustrated by the equation:

$$\text{Acetic acid} + O_2 \longrightarrow \text{Stored carbohydrates} + CO_2 + H_2O.$$

It could be assumed that the over-all reaction represents a summation of exergonic and endergonic reactions linked by the energy transfer between them in a manner similar to the mechanisms of chemosynthesis in *Thiobacillus thiooxidans* (Vogler and Umbreit, **19**).

Among the mechanisms suggested for the organic fixation of carbon dioxide, the carboxylation of pyruvic acid is of interest because it is directly related to carbohydrate metabolism. The carboxylation of pyruvate has been held difficult to accomplish because of the energy relations ($\Delta F = -15.2$ in aqueous solution and $\Delta F = -2.9$ in alkaline solution) involved. Carson, Ruben, Kamen, and Forster (**6**) were unable to produce this reaction. Van Niel *et al.* (**18**) suggested that these carboxylations are necessary for growth and respiration because they contribute to the synthesis of 4-carbon acids (fumaric, malic, succinic, and oxalacetic), which play an important part in respiration; also, perhaps, in photosynthesis.

Krampitz and Werkman (**12**) and Krampitz, Wood, and Werkman (**13**) were able to demonstrate reversible carboxylation of oxalacetic acid.

BONNER and BONNER (**4**) found that an increase of carbon dioxide concentration to bryophyllum in the dark under controlled conditions produced a definite increase in total acids. The total acids were fully accounted for by totalling the citric, isocitric and cis-aconitic, and the malic acids fractions. Bonner and Bonner state, "The other acids necessarily present in a plant respiration cycle may be present in such small quantities that available analytical methods are inadequate to detect them. They may be overshadowed by the large amount of the acids determined . . . . The fact that the increase in malate is a direct function of carbon dioxide pressure suggests that at least a portion of the oxalacetic acid formed is at once reduced to malic acid. On the other hand, isocitric accumulation with an increase of carbon dioxide would indicate that a part of the oxalacetate is removed by oxidative transformation."

KROTKOV and BARKER (**14**) using C^{14} in the carboxyl group of acetic acid found that tobacco leaves could utilize acetate in the dark. When tobacco leaves were left for three hours and five minutes in the dark, after having been placed in acetic solution for about two hours, the C^{14} was located in an unidentified water and ethanol soluble compound. When, after being placed in acetate solution for about two hours, the leaves were left in darkness for 20 hours and 45 minutes, the isotopic carbon appeared mainly as respired CO_2. In both cases, acetate was metabolized. The results strongly suggest that, once the carbohydrate supply of the leaves is exhausted, acetic acid is used directly for the formation of cis-aconitic acid in endogenous respiratory processes.

Recently MYERS, CRAMER, and JOHNSTON (**15**) reported an oxidative assimilation of acetic acid in darkness by the green algae, *Chlorella pyrenoidosa*.

Basing their conclusions on the results of experiments with isotopic carbon dioxide, ALLEN, GEST, and KAMEN (**1**), and GAFFRON, FAGER, and BROWN (**10**) believed that starved chlorella cells were able to fix carbon dioxide by endogenous respiration. This is definitely shown in the data presented here by the good correlation existing between the utilization of the organic acids in the dark, the increase of oxygen uptake and the low respiratory quotient produced by them.

Using isotopic carbon and yeast, WEINHOUSE and MILLINGTON (**20**) observed that acetate produced an accumulation of citrate and of carbon dioxide which was distributed within the tricarboxylic acid cycle. They state, however, that another independent mechanism is available for the formation of some of the 4-carbon acids from acetic acid. KALNITSKY and WERKMAN (**11**) demonstrated that succinate is not produced in some organisms through the decarboxylation of α-ketoglutarate but by the condensation of two acetates. CALVIN and BENSON (**5**) believe that this oxidative coupling of two acetates to produce succinate also occurs in chlorella. In the present study, the low rate of respiration observed with succinate tends

to confirm the previous indications that succinate enters an independent mechanism and is not a direct intermediate in the aerobic cycle of chlorella.

Summary

The effect of various organic acids known to occur in the path of carbohydrate metabolism in animal tissues was studied on the respiration of the green alga chlorella. Manometric measurements of oxygen uptake and carbon dioxide evolution were conducted in the dark with a Warburg apparatus. The following acids were supplied: lactic, pyruvic, acetic, aconitic, citric, α-ketoglutaric, succinic, fumaric, malic, butyric, and propionic.

Respiration was stimulated in various degrees by the organic acids and their concentrations. Pyruvate and acetate produced the highest oxygen uptake and citrate and succinate the lowest, correlating well with the growth results (**9**). The apparent respiratory quotient for most of the acids was lower than unity. The relation between the oxygen uptake and carbon dioxide evolution, together with the growth results, indicate the formation of reduced compounds through oxidative assimilation and carbon dioxide fixation. The fact that the organic acids utilized for respiration and growth by chlorella are identical to those known as intermediates in the respiratory path of animal tissues indicates that the path through which the oxidation of carbohydrates is effected has much similarity with the tricarboxylic acid cycle and that it can reverse itself in the dark to build organic materials necessary for growth from organic acids and carbon dioxide.

It is a pleasure to acknowledge the suggestions of Drs. Lewis Knudson and H. H. Williams during the course of this investigation. Thanks are also due to Dr. Gordon Hunt for his help and to Dr. Betsy Smith for donating the α-ketoglutaric acid.

CORNELL UNIVERSITY
ITHACA, NEW YORK

LITERATURE CITED

1. ALLEN, M. B., GEST, H., and KAMEN, M. D. Differential inhibition of respiration and dark carbon dioxide fixation in Scenesdesmus and Chlorella. Arch. Biochem. **14**: 335–347. 1947.
2. ANDERSON, EDWARD H. The metabolism of the colorless alga *Prototheca zopfii*. Jour. Gen. Physiol. **28**: 297–327. 1945.
3. BARKER, H. ALBERT. The metabolism of the colorless alga *Prototheca zopfii* Krüger. Jour. Cellular & Comp. Physiol. **7**: 73–93. 1935.
4. BONNER, WALTER and BONNER, JAMES. The role of carbon dioxide in acid formation by succulent plants. Amer. Jour. Bot. **35**: 113–117. 1948.
5. CALVIN, M. and BENSON, A. A. The path of carbon in photosynthesis. Science **107**: 476–480. 1948.
6. CARSON, S. F., RUBEN, S., KAMEN, M. D., and FORSTER, J. W. Radio-

active carbon as an indicator of carbon dioxide utilization. VI. The possibility of carbon dioxide reduction via the carboxylase system. Proc. Natl. Acad. Sci. **27**: 475–480. 1941.

7. CLIFTON, CHARLES E. Possibility of preventing assimilation in respiring cells. Enzymologia **4**: 246–253. 1937. Nature **140**: 318–319. 1937.
8. DOUDOROFF, MICHAEL. Utilization and synthesis of sucrose and related compounds by some microorganisms. Federation Proc. **4**: 241–247. 1945.
9. ENY, DÉSIRÉ M. Respiration studies on chlorella. I. Growth experiments. Plant Physiol. **25**: 478–495. 1950.
10. GAFFRON, H., FAGER, E. W., and BROWN, A. Report on isotope symposium. U. of Wisconsin. 1947.
11. KALNITSKY, GEO. and WERKMAN, CHESTER H. The anaerobic dissimilation of pyruvate by a cell-free extract of *Escherichia coli*. Arch. Biochem. **2**: 113–124. 1943.
12. KRAMPITZ, LESTER O. and WERKMAN, CHESTER H. The enzymic decarboxylation of oxaloacetate. Biochem. Jour. **35**: 595–602. 1941.
13. KRAMPITZ, L. O., WOOD, H. G., and WERKMAN, C. H. Enzymic fixation of carbon dioxide in oxalacetate. Jour. Biol. Chem. **147**: 243–253. 1943.
14. KROTKOV, G. and BARKER, H. A. Utilization of acetate by tobacco leaves, as determined with C^{14}. Amer. Jour. Bot. **35**: 12–15. 1948.
15. MYERS, JACK, CRAMER, MARIAN, and JOHNSTON, JAMES. Oxidative assimilation in relation to photosynthesis in chlorella. Jour. Gen. Physiol. **30**: 217–227. 1947.
16. RUBEN, S., HASSID, W. Z., and KAMEN, M. D. Radioactive carbon in the study of photosynthesis. Jour. Amer. Chem. Soc. **61**: 661–663. 1939.
17. UMBREIT, W. W., BURRIS, R. H., and STAUFFER, J. F. Manometric techniques. Burgess Publ. Co., Minneapolis. 1947.
18. VAN NIEL, C. B., RUBEN, S., CARSON, S. F., KAMEN, M. D., and FORSTER, J. W. Radioactive carbon as an indicator of carbon dioxide utilization. VIII. Role of carbon dioxide in cellular metabolism. Proc. Natl. Acad. Sci. **28**: 8–15. 1942.
19. VOGLER, K. G. and UMBREIT, W. W. Studies on the metabolism of autotrophic bacteria. III. The nature of the energy-storage material active in the chemosynthetic process. Jour. Gen. Physiol. **26**: 157–167. 1942.
20. WEINHOUSE, SIDNEY and MILLINGTON, RUTH H. Acetate metabolism in yeast, studied with isotopic carbon. Jour. Amer. Chem. Soc. **69**: 3089–3093. 1947.
21. WOLF, JOHANNES. Oxygen metabolism in the succulent Crassulaceae. V. Microrespiration of leaf tissues of *Bryophyllum calycinum*. Planta **29**: 450–467. 1939.

EFFECT OF VARIOUS FACTORS ON THE UTILIZATION OF NITROGEN AND IRON BY *SPIRODELA POLYRHIZA* (L.) SCHLEID.[1]

EZRA H. BITCOVER AND DALE H. SIELING

Received October 30, 1950

Introduction

The importance of nitrogen in the nutrition of green plants and the role of iron in the development of green plants have long been established. The interrelationship between iron and nitrogen in the metabolism of green plants is not clearly understood, however. A clue to this interrelationship might be obtained by determining the effects of a few variables on the utilization of nitrogen and iron.

Most investigations concerning the uptake of different forms of nitrogen were made in non-sterile media so that the results observed were often of doubtful accuracy as to form of nitrogen actually assimilated by green plants. Much work has been done in studying the uptake of various forms of nitrogen, particularly ammonium and nitrate. Relatively little research has been done, however, in studying the uptake of organic forms of nitrogen. It would be of fundamental interest to establish what forms and what amounts of nitrogen are assimilated by green plants.

The optimum amount of iron for growth of green plants has never been adequately established. The use of a "pinch" or "trace" of an iron salt in nutrient solutions has furnished sufficient iron for plant growth, but the concentration and form of the available iron has not been known. In acidic nutrient solutions toxic excess amounts of iron were often encountered, and in neutral and basic nutrient solutions insufficient amounts of iron were available for plant growth. It would be of value to establish an optimum range of concentrations for iron for ordinary conditions of plant growth and to determine in what chemical forms such iron must be in order to be utilizable by green plants.

In this series of experiments, *Spirodela polyrhiza* (L.) Schleid. (called *Lemna major* or duckweed by some writers) was used. This common pond plant may be easily grown under aseptic conditions, thus eliminating all possibility of erroneous results due to the presence of extraneous organisms. The precise control of the environment permits exact quantitative work capable of duplication. According to STEINBERG (**23**, **24**, **25**), the nutrient requirements of Spirodela appear to be quite similar to those of other green plants, and the results obtained through its use may be directly checked with different crop plants.

[1] Contribution No. 782, Massachusetts Agricultural Experiment Station, Amherst, Mass.

Historical

BEAUMONT (2), KLEIN (13), and others (4, 13, 20), have established that plants may directly assimilate such forms of nitrogen as nitrate, ammonium, urea, and certain amino acids. Various amino acids are absorbed by plants, but are not equally utilizable. GHOSH and BURRIS (8) have reported recently that certain organic nitrogen compounds may be superior to inorganic sources of nitrogen for plants. BRIGHAM (4) pointed out, however, that organic compounds of high complexity in composition are much more effective for plant growth after ammonification.

BEAUMONT (1) and others have reported toxicity resulting from the use of ammonium compounds. According to PRIANISHNIKOV (17, 19) plants absorb ammonium actively as long as the reaction of the medium does not hinder its immediate transformation into organic nitrogenous combinations. In neutral or slightly alkaline media, ammonium produces better growth results, while in acid media nitrate is a better source of nitrogen for plants. PRIANISHNIKOV (18) claimed that ammonium enters plants faster than nitrates, and is more easily assimilated. With excess ammonium, injury appears more readily than with excess nitrates. Thus, nitrification regulates the concentration of ammonium so that ammonium toxicity can only rarely occur in soils. Ammonium is the principal material serving for the synthesis of organic nitrogenous materials by combining with organic acids which arise from the oxidation of carbohydrates to form amino acids. Nitrates must first be reduced before the nitrogen can be utilized in forming organic nitrogenous combinations.

According to BENNETT (3) chlorosis is brought about by a disturbance of the nitrogen metabolism as well as of the iron metabolism in plants. The physiological functions of the two elements are intimately related. Many investigators have reported that the use of organic acid salts of iron such as citrate, tartrate, and oxalate increased the amount of iron in solution, particularly in the alkaline range. It was reported as early as 1895 (10) that organic sources of iron produced better results in correcting chlorosis than inorganic sources of iron. GILE and CARRERO (9) reported in 1916 that ferrous sulphate, ferric citrate, and ferric tartrate afforded sufficient iron for plant growth in acid, neutral, and alkaline solutions when used in proper quantities, but that ferric chloride was an inferior source of iron. FLY (7) reported that when ferric chloride was used as the iron source, there was not sufficient iron available for plant growth above pH 5, but that when ferric citrate was used, there was sufficient iron in solution to support the growth of Spirodela up to pH 8. HOPKINS and WANN (11, 12) found that the addition of sodium citrate to culture solutions maintained sufficient iron in solution even at alkaline reactions. SIELING (21) also reported that a humic acid solution of iron was effective in preventing chlorosis of Spirodela in neutral and alkaline solutions. OLSEN (16) offered the hypothesis that iron is taken up from neutral or alkaline solutions and is precipitated in the vascular bundles as ferric phosphate, but that ferric citrate does not give

up its iron to the phosphate, and the iron thus passes through the plant as citrate.

Materials and methods

In this series of experiments the absorption of urea, guanidine, ammonium, and nitrate forms of nitrogen was studied, as well as the effect of citric acid and iron on the assimilation of ammonium and nitrate.

The test plants were sterilized by soaking them in a 0.5% solution of NaOCl for 60 seconds, following which they were rinsed twice with sterile distilled water. The plants were checked for sterility by incubating one plant from each culture in Bacto-nutrient broth at 37° C for 24 to 48 hours, and checking for the presence of contaminant organisms as shown by turbidity.

The test plants were grown in 300-ml. Erlenmeyer flasks containing complete nutrient solutions in a growth chamber especially constructed for the purpose (**6**). The flasks were placed in a water bath which was maintained at 25° C ± 1° C by thermostat. Constant illumination of 500 fc at the surface of the plants was provided by six 40-watt fluorescent lamps for 15 hours a day. Transfers of plants from one flask to another were performed under aseptic conditions. The plants were transferred to fresh solutions every fourth day. Duplicate cultures were grown in every instance and all data presented are averages of the duplicates. In every case the variation between duplicate cultures was insignificant.

Complete nutrient solutions were used in which the source of nitrogen was varied in each solution. Table I shows the composition of the nutrient solutions. It was found necessary to sterilize the urea, the ammonium sulphate, and the potassium hydroxide solutions separately from the remaining constituents of the nutrient solutions in order to obtain the desired pH values after sterilization. Sterilization was accomplished by autoclaving at a steam pressure of 15 pounds for 15 minutes.

When conditions are suitable for growth and are kept uniform, the rate of increase of Spirodela at any time is proportional to the number of plants present, according to CLARK (**5**) and WHITE (**27**), and is represented by the equation $\log_{10}\frac{N}{N_o} = K(t - t_o)$, where N = number of plants at time t. When the log of the number of plants at any time is plotted against time, the result is a straight line as shown by CLARK (**5**). The slope of the line is proportional to the effectiveness of the treatment. This effectiveness may be represented by what are hereinafter referred to as Σ values. The Σ values recorded in the data refer to the summation of the $(\log N - \log N_o)$ values from the start of any experiment to the 24th day.

Results

PART I. EFFECT OF CONCENTRATION OF NITROGEN.—In order to determine the effect of varying the concentration of nitrogen, a series of experiments

TABLE I

COMPOSITION OF THE NUTRIENT SOLUTIONS*

	Molarity	gm./l.	mg. N/l.
Nitrate Solution			
$CaH_4(PO_4)_2 \cdot H_2O$	.0002	.0504	92
KNO_3	.0065	.6565	
$MgSO_4 \cdot 7H_2O$	.0005	.123	
Ammonium Solution			
$(NH_4)_2SO_4$	.0033	.430	92
K_2SO_4	.0033	.575	
$MgSO_4 \cdot 7H_2O$	.0005	.123	
$CaH_4(PO_4)_2 \cdot H_2O$	.0002	.0504	
Urea Solution			
Urea (3.0030 g/l.)	.0033	.197	92
K_2SO_4	.0033	.575	
$MgSO_4 \cdot 7H_2O$	.0005	.123	
$CaH_4(PO_4)_2 \cdot H_2O$	.0002	.0504	
Guanidine Solution			
Guanidine . HCl (4.7765 g/l.)	.0022	.209	92
K_2SO_4	.0033	.575	
$MgSO_4 \cdot 7H_2O$	.0005	.123	
$CaH_4(PO_4)_2 \cdot H_2O$	.0002	.0504	

*Stock solutions of each of the salts were provided in .05 M. and .01 M. concentrations. Boron and manganese salts, sufficient to provide 1 p.p.m. of these elements in the final volume, and enough of Hoagland's A-Z solution to provide .01 to .02 p.p.m. of the minor elements were added before dilution. Five p.p.m. iron as ferric citrate was provided. The solutions were adjusted to the desired pH values with sterile sulphuric acid or potassium hydroxide.

was performed in which the concentrations of nitrogen used were 92, 46, and 9 mg. per liter. Different solutions were adjusted to pH values 5.0, 6.0, 7.0, and 8.0. The results obtained from these experiments are summarized in table II.

It is obvious that nitrate was the best source of nitrogen at the highest concentration tried. The plants of the other series grew very poorly at this concentration, the ammonium being so toxic that no growth data could be obtained. These series produced plants which were small, chlorotic, yellowish, white, or scorched at the edges of the fronds in different cases. The roots dropped off the fronds in some solutions. In other solutions, particularly the ammonium series, the fronds existed as individuals, rather than in colonies of two, four, or more fronds as in the normal healthy plants. In the nitrate series, the plants grew almost equally well at pH values of 6.0, 7.0, and 8.0; less well at pH 5.0. It was not possible to maintain growth in solution at pH 4.0 with any of the nitrogen sources tested.

At a concentration of 46 mg. nitrogen per liter nitrate was still the best source of nitrogen. The growth of plants in the urea and ammonium series

TABLE II

EFFECT OF CONCENTRATION OF NITROGEN ON GROWTH OF *Spirodela polyrhiza* AT VARIOUS PH VALUES

	pH	Σ Value 24 days	Remarks
			92 mg. nitrogen per liter*
Nitrate	5	2.05	pale green
	6	2.24	normal green
	7	2.26	normal green
	8	2.26	yellowish green
Ammonium	5		no growth
	6		no growth
	7		no growth
	8		no growth
Urea	5	.70	tiny, pale
	6	.25	tiny, chlorotic
	7	.49	small, pale
	8	1.19	normal size, pale
Guanidine	5	.85	tiny, unhealthy dark green, speckled purple undersides
	6	.24	small, chlorotic, speckled purple undersides
	7	.36	normal size, chlorotic, speckled purple undersides
	8	.17	normal size, chlorotic, speckled purple undersides
			46 mg. nitrogen per liter
Nitrate	5	2.32	normal, green
	6	2.45	normal, green
	7	2.35	large, green
	8	2.71	large, pale green
Ammonium	5	2.22	small, wrinkled fronds
	6	2.50	small, wrinkled fronds
	7	2.22	normal size, yellowish
	8	1.20	normal size, yellowish
Urea	5	1.07	tiny, unhealthy dark green
	6	.25	small, chlorotic
	7	1.54	small, pale green
	8	1.89	normal size, green
Guanidine	5	1.34	tiny, speckled purple undersides
	6	.20	small, chlorotic, speckled purple undersides
	7	.28	normal size, speckled purple undersides
	8	.21	normal size, speckled purple undersides
			9 mg. nitrogen per liter
Nitrate	5	1.74	small, green
	6	1.75	normal size, green
	7	1.92	normal size, green
	8	1.82	normal size, pale green
Ammonium	5	1.72	small, wrinkled fronds
	6	2.12	normal size, individual fronds
	7	1.86	normal size, light green
	8	1.82	large, light green
Urea	5	1.92	tiny, pale green
	6	.99	tiny, yellowish green
	7	1.75	small, pale green
	8	1.70	normal size, pale green
Guanidine	5	1.05	chlorotic, speckled purple undersides
	6	1.45	chlorotic, speckled purple undersides
	7	1.27	yellowish, speckled purple undersides
	8	1.23	unhealthy dark green, speckled purple undersides

*Composition of nutrient solutions are given in table I for 92 mg. nitrogen per liter. In other solutions of lower nitrogen concentration the amount of material furnishing the nitrogen was reduced, but the composition of the balance of the solutions was kept constant.

appeared better at 46 than at 92 mg. nitrogen per liter. Although the Σ value for the nitrate solution was higher at pH 8.0 than at pH 7.0, the plants appeared to be healthier at pH 7.0. Even though the plants in the ammonium series reproduced well, their appearance was rather unhealthy (small, chlorotic, or wrinkled) when compared with the nitrate series. The plants in the urea series appeared better than in the ammonium series, but were also rather unhealthy, except at the higher pH's, 7.0 and 8.0.

When the concentration of nitrogen was reduced to about one-tenth the maximum concentration tested, the nitrate series plants did not appear as healthy as at the higher concentrations, but were somewhat paler. In fact, the ammonium series plants seemed better (deeper green) than the nitrate series. It was also noticed that pH 7.0 was the best pH for both the nitrate and ammonium series. Even though the Σ value in the ammonium series was greater at pH 6.0 than at pH 7.0, the general appearance of the plants was much superior at pH 7.0.

The plants of the urea series were somewhat intermediate in their growth and appearance between the ammonium and nitrate series. In the urea series growth was poorest at pH 6.0 for all the nitrogen concentrations used.

The growth of the plants of the guanidine series varied indirectly with concentration. Guanidine as a source of nitrogen compared unfavorably with nitrate, ammonium, and urea. There was a marked degree of purpling on the undersides of the fronds which appeared first as specks or flecks, and then increased in size as the plants were maintained in the culture solutions. The degree of purpling increased as the guanidine concentration decreased so that the undersides of the fronds were often entirely purpled.

PART II. EFFECT OF IRON AND CITRIC ACID.—In the experiments of Part I, 5 p.p.m. of iron as ferric citrate were used as the source of iron. The assimilation of nitrogen by plants is influenced, however, by the availability of iron, which in turn is affected by the reaction (pH) of the solution and the concentration of the complexing anion. In any study of nitrogen assimilation, it is therefore necessary that the amounts of available iron be in the range of optimum concentration. It therefore seemed desirable to determine the effects of various combinations of iron and complexing anion (citrate in this case) upon the utilization of iron and nitrogen. A series of experiments was performed in which the concentration of iron was varied, and the molar ratio of citric acid to iron was varied. The nutrient solutions were adjusted to pH values 5.0, 6.0, 7.0, and 8.0. A concentration of 9 mg. nitrogen per liter was selected because it had been found that the ammonium and nitrate series plants grew approximately equally well at this concentration.

Quantitative colorimetric test (**22**) showed that less than one part iron per 100,000,000 was present as an impurity in the nutrient solutions as prepared. It was also established by the use of nitrate solutions at concentrations of 92 mg. and 46 mg. nitrogen per liter, without the addition of iron, at pH 6.0, that the rate of growth of Spirodela was greatly retarded. The

TABLE III

EFFECT OF MOLAR RATIO OF CITRIC ACID TO IRON ON THE GROWTH OF *Spirodela polyrhiza* AT 2 p.p.m. IRON AND 9 p.p.m. NITROGEN*

pH	Σ Value 24 days	Remarks
		Nitrate (1 : 1 ratio)
5	1.38	small, yellowish, fine reddish sediment
6	1.22	small, light-green, fine reddish sediment
7	1.84	normal size, pale green, fine reddish sediment
8	1.66	normal size, yellowish, fine reddish sediment
		Ammonium (1 : 1 ratio)
5	2.16	small, wrinkled fronds, fine reddish sediment
6	1.92	small, yellowish green, fine reddish sediment
7	1.88	normal size, light green, fine reddish sediment
8	1.90	normal size, pale green, fine reddish sediment
		Nitrate (2 : 1 ratio)
5	1.44	small, light green
6	1.72	normal size, light green
7	2.12	normal size, light green
8	1.95	normal size, pale green
		Ammonium (2 : 1 ratio)
5	2.14	small, wrinkled fronds
6	1.85	normal size, yellowish green
7	2.00	normal size, pale green
8	1.95	normal size, pale green
		Nitrate (4 : 1 ratio)
5	1.50	small, light green
6	1.94	normal size, green
7	2.28	normal size, green
8	2.36	normal size, yellowish green
		Ammonium (4 : 1 ratio)
5	1.90	small, wrinkled fronds
6	2.30	normal size, pale green
7	2.25	normal size, green
8	2.26	large, light green
		Nitrate (8 : 1 ratio)
5	1.98	small, yellowish green
6	1.54	small, pale green
7	2.00	small, light green
8	2.11	normal size, pale green
		Ammonium (8 : 1 ratio)
5	2.15	small, wrinkled fronds
6	2.15	small, light green
7	2.15	normal size, pale green
8	2.15	normal size, pale green

*Composition of solutions the same as given in table I except that the component which furnished the nitrogen was reduced to 1/10 and the ferric citrate was omitted. $FeCl_3 . 6H_2O$ and citric acid added to fix those concentrations and ratios.

TABLE IV

EFFECT OF CONCENTRATION OF IRON ON THE GROWTH OF *Spirodela polyrhiza* AT A 4:1 MOLAR RATIO OF CITRIC ACID TO IRON AND 9 p.p.m. NITROGEN*

pH	Σ Value 24 days	Remarks
	Nitrate (10 p.p.m. Fe)	
5	.62	small, yellowish
6	.98	normal size, pale green
7	1.90	normal size, light green
8	1.86	normal size, light green
	Ammonium (10 p.p.m. Fe)	
5	.60	small, chlorotic
6	1.24	small, pale green
7	1.98	normal size, green
8	1.80	normal size, green
	Nitrate (20 p.p.m. Fe)	
5	.04	whitish
6	.23	small chlorotic
7	.74	small, chlorotic
8	1.88	small, pale green
	Ammonium (20 p.p.m. Fe)	
5		died
6	.32	small, chlorotic
7	.94	small, pale green
8	2.24	normal size, green
	Nitrate (40 p.p.m. Fe)	
5		died
6	.31	whitish
7	.74	chlorotic
8	1.88	normal size, green
	Ammonium (40 p.p.m. Fe)	
5	.21	whitish
6	.55	small, chlorotic
7	1.76	small, yellowish, speckled brown
8	1.54	small, green
	Nitrate (80 p.p.m. Fe)	
5		died
6	.05	white, died
7	.42	tiny, chlorotic
8	1.14	small, yellowish, scorched edges
	Ammonium (80 p.p.m. Fe)	
5		died
6	.14	whitish
7	1.10	tiny, yellowish
8	.42	tiny, scorched

*Solutions same as shown in table III.

plants became chlorotic in less than a week, indicating that insufficient iron for plant growth was present as an impurity in the reagent chemicals.

In the first experiment a concentration of 2 p.p.m. of iron as $FeCl_3 \cdot 6H_2O$ was used, and amounts of citric acid were selected so that molar ratios of citric acid to iron were 1 to 1, 2 to 1, 4 to 1, and 8 to 1. The results of this experiment are summarized in table III. It appeared that the 4 to 1 citric acid : iron ratio was the best ratio for both the ammonium and nitrate series based on general vigor of the plants and the Σ values. In the 1 to 1 series a fine reddish brown sediment was noticed on the bottom of each flask. This sediment was not present at the higher ratios. At all the ratios, the plants in the ammonium series seemed to grow better than those in the nitrate series. The growth of Spirodela at pH 7.0 was very similar, however, in both the ammonium and nitrate series.

Since the optimum iron concentration for the growth of Spirodela was not known, growth experiments were carried out at concentrations of 10, 20, 40, and 80 p.p.m. of iron, using a 4 to 1 citric acid : iron ratio. The results in table IV show that a concentration of 10 p.p.m. of iron produced the best growth under these conditions. At concentrations of 40 p.p.m. of iron and above, growth was greatly retarded. The best growth at all concentrations took place at pH's 7.0 and 8.0. The general appearance and growth of the

TABLE V

EFFECT OF MOLAR RATIO OF CITRIC ACID TO IRON ON THE GROWTH OF *Spirodela polyrhiza* AT 10 p.p.m. IRON AND 9 p.p.m. N*

pH	Σ Value 24 days	Remarks
	Nitrate (16 : 1 ratio)	
5	1.40	very small, yellowish
6	1.82	small, pale
7	1.72	normal size, very pale
8	1.91	normal size, very pale
	Ammonium (16 : 1 ratio)	
5	1.16	very small, yellowish
6	1.89	small, very pale
7	1.93	small, very pale
8	1.82	small, very pale
	Nitrate (32 : 1 ratio)	
5	1.44	tiny, yellowish, speckled brown
6	1.42	tiny, yellowish, speckled brown
7	.25	small, yellowish
8	.08	small, chlorotic
	Ammonium (32 : 1 ratio)	
5	.90	small, pale
6	.68	small, pale
7	.20	small, chlorotic
8	.28	small, chlorotic, individuals

* Solutions same as shown in table III.

plants, however, was best at a concentration of 2 p.p.m. This is found by comparing the Σ values for the 4 to 1 ratios of tables III and IV.

As a test of the hypothesis of HOPKINS and WANN (12) that increasing the citrate content of the nutrient solution decreases the ionization of the ferric citrate present so that growth is retarded because of a low concentration of Fe^{+++} in solution, the following experiment was performed. A concentration of iron equal to 10 p.p.m. was selected, and citric acid : iron ratios of 16 to 1 and 32 to 1 were prepared. A concentration of 10 p.p.m. of iron was selected because it was thought that 2 p.p.m. might not furnish adequate iron for plant growth at the high citric acid : iron ratios used. On the basis of size and color of the plants the results in table V show that at the 16 to 1 ratio growth was retarded by the relatively high concentration of citrate, and that the plants in the nitrate series were better than those in the ammonium series. At the 32 to 1 ratio the growth of the Spirodela plants was very greatly retarded by the high concentration of citrate at all pH values, and the fronds were very small, and pale, yellowish or chlorotic.

Discussion

The results obtained in Part I seem to indicate that the effectiveness of any form of nitrogen in promoting growth is influenced primarily by concentration. When the reaction of the medium was not extreme (*i.e.*, pH 5.0 to 8.0), nitrate nitrogen seemed to be almost equally efficient over a wide range of concentrations in promoting growth, provided, of course, that it was not present to a toxic excess or in insufficient amounts. Nitrate nitrogen was quite effective as a source of nitrogen at all concentrations but ammonium nitrogen appeared to be toxic at the higher concentrations; however, at the lowest concentration, ammonium appeared to be a more efficient form of nitrogen than nitrate. It thus seems that plants are able to tolerate and utilize nitrogen from higher concentrations of nitrate than of ammonium at moderate reactions, and that much lower concentrations of ammonium nitrogen than of nitrate nitrogen are needed to produce excellent growth.

Urea nitrogen was intermediate between nitrate and ammonium in its effects on the growth of Spirodela. Urea is apparently more efficient at neutral and slightly alkaline reactions. It is well known that urea is readily absorbed by green plants. The fact that nitrogen is present as NH_2 in the urea molecule would seem to indicate its easy absorption and assimilation. The ammonium form itself is toxic above certain low concentrations. This may be a clue to the question as to why the Spirodela plant can tolerate higher concentrations of urea than of ammonium. The fact that Spirodela can tolerate higher concentrations of nitrate than of urea may be explained by the fact that plants absorb and store relatively large amounts of nitrate, reducing only such amounts as they require for assimilation. Urea, having the NH_2 form, is immediately available for assimilation, and in that form plants can only utilize certain amounts without toxic results.

Guanidine was found to be inefficient as a nitrogen source for the growth

of Spirodela at all concentrations used, although growth was promoted at the lowest concentration. Purpling of the fronds increased as the guanidine concentration decreased. This purpling may be an indication of some unbalanced condition in the nitrogen metabolism of the plant. Such purpling of the fronds is often noticed in ponds containing *Spirodela polyrhiza*, particularly in midwestern United States.

At a nitrogen concentration at which ammonium and nitrate were about equally effective in maintaining growth ammonium and nitrate were approximately equally efficient in neutral solutions; however, nitrate was somewhat more effective below neutrality (pH 7.0) and ammonium above neutrality.

The results in Part II suggest that the addition of an organic acid, such as citric acid, will maintain sufficient iron available in solution for plant growth, even in alkaline solutions. It was found, however, that there must be more than a molar equivalence of citric acid to iron to maintain all the iron in solution. STRUTHERS and SIELING (**26**) reported that more than one mole of citric acid is required per mole of iron to maintain all the iron in solution. This fact was substantiated in these experiments. However, it was found that a higher mole ratio, *viz.*, 4 to 1, promoted more effective growth. Subsequent work in this laboratory has shown that in dilute solutions one mole of citric acid will maintain one mole of iron in solution between pH 4.0 and pH 6.0, but that citric acid is somewhat less effective at higher and lower pH values.

LANFORD and QUINAN (**15**) have found evidence that iron forms a complex with citric acid whose formula may be represented as $Fe(H\ citr)^{+}$ and its ionization is represented by:

$$Fe(H\ citr)^{+} \rightleftharpoons Fe^{+++} + H\ citr^{--}$$

This formula is of the same type as that for the complex formed by the reaction of ferric iron with phosphoric acid, which has been shown to be $FeHPO_4^{+}$ (**14**). This suggests that the form in which iron is taken up by plants may be ferric organic complex ion instead of Fe^{+++} as has been widely accepted, and that $Fe(H\ citr)^{+}$ is probably the form absorbed when citrates are present. Although it is known that green plants absorb both ferrous and ferric ions quite effectively in the absence of organic chelating compounds from the nutrient solution, the iron is probably complexed by organic substances present in the root epidermal cells, and thus enters the plant as ferric organic complex ion. Work in this laboratory has shown that pectic and uronic acids (compounds which are among those present in roots) are capable of forming stable complex ions with iron.

This does not mean to imply that Fe^{+++} would not be utilizable by plants, but that there is not enough Fe^{+++} in solution at pH values near neutrality to supply sufficient iron for plant growth. Since the solubility product of $Fe(OH)_3$ is 1×10^{-36}, the concentration of Fe^{+++} at pH 7.0 is 1×10^{-15} molar or 5.5×10^{-11} p.p.m. and chlorosis usually develops unless a complex-forming

organic anion is present. Thus, an excess of complexing agent may repress the ionization of the ferric organic complex ion to such an extent that the plant would be unable to obtain sufficient Fe^{+++} from the complex ion.

In comparing the Σ values in tables III, IV, and V, it would seem that the optimum concentration of iron for the growth of Spirodela is 2 p.p.m. and the optimum ratio of citric acid : iron is 4 to 1. It is possible, however, that a much lower concentration of iron as $Fe(H\ citr)^{+}$ is adequate for maintaining good growth of Spirodela. The concentration of $Fe(H\ citr)^{+}$ in the various nutrient solutions was not determined.

The experimental results indicate that at a concentration of 2 p.p.m. of iron, a molar ratio of citric acid to iron of 1 to 1 was insufficient to maintain all the iron in solution. When the ratio was increased to 2 to 1, healthier growth resulted, but the 4 to 1 ratio produced the best growth based on appearance and Σ values. This would seem to indicate that at the 2 to 1 ratio all the iron was in solution as far as it was possible to observe, although the chemical forms were difficult to determine. The chemical conditions within the nutrient solutions were such that the amount of citric acid required to furnish the optimum amount of $Fe(H\ citr)^{+}$ was not present until the 4 to 1 ratio was used.

Work in this laboratory has shown that the $Fe\ (H\ citr)^{+}$ complex is most stable between pH values 4.0 and 6.0, since more $(H\ citr)^{--}$ is present. This fact can explain the growth results reported in tables III, IV, and V. The best growth took place in the more basic solutions, and the poorest growth in the more acid solutions. This suggests that the $Fe(H\ citr)^{+}$ concentration was somewhat excessive in the acid solutions, but that it was present in "safe" amounts in the basic solutions. The addition of (OH) ions seems to reduce the concentration of $Fe(H\ citr)^{+}$ because it changes the $(H\ citr)^{--}$ to $(citr)^{---}$ and reduces the $Fe(H\ citr)^{+}$ form to a "safe" level for plant growth. In the case of the higher ratios either the concentration of citrate, or complex ion, or iron itself, becomes toxic.

Summary and conclusions

Spirodela polyrhiza plants were grown in sterile culture solutions under conditions of constant temperature and illumination. The composition of the culture solutions was similar in all cases except that the form of nitrogen was different. The uptake of nitrogen as guanidine, urea, ammonium, and nitrate was studied under various conditions of pH and concentration of nitrogen. The effect of iron and an organic acid (citric acid) on the uptake of ammonium and nitrate under various conditions of pH and various molar ratios of citric acid to iron was also studied.

The results indicate that the effectiveness of any form of nitrogen in promoting growth is influenced primarily by concentration. Nitrate was a more effective source of nitrogen than any other form of nitrogen studied over a greater range of concentrations. Ammonium nitrogen was toxic at the higher concentrations where nitrate was very effective in promoting

growth; however, at much lower concentrations, ammonium was more efficient in promoting growth than nitrate. Urea was intermediate in its effect on plant growth between ammonium and nitrate. Guanidine was inefficient in promoting growth at all the concentrations used in these experiments, and caused a purpling of the undersides of the fronds which increased as the guanidine concentration decreased in the culture solutions.

A citric acid to iron ratio greater than 1 to 1 maintained all the iron in solution, even in basic solutions. The optimum ratio for the growth of *Spirodela polyrhiza* was 4 to 1. The optimum total concentration of iron was 2 p.p.m. Growth was greatly retarded above 20 p.p.m. of iron and a 16 to 1 ratio. The best growth took place in the basic solutions, and the poorest growth in the acidic solutions.

It is postulated on the basis of the data of these experiments that iron enters the plant as ferric organic complex ion, or is transported to the plant root where it is utilized as Fe^{+++} subsequent to its release from the complex ion.

Department of Agronomy
University of Massachusetts
Amherst, Massachusetts

LITERATURE CITED

1. Beaumont, A. B. The toxicity of ammonium compounds for tobacco. Proc. Sec. Int. Cong. Soil Sci. Pp. 65–78. 1930.
2. Beaumont, A. B. *et al.* The assimilation of nitrogen by tobacco. Jour. Agr. Res. **43**: 559–567. 1931.
3. Bennett, J. P. Iron in leaves. Soil Sci. **60**: 91–105. 1945.
4. Brigham, O. Assimilation of organic nitrogen by *Zea mays* and the influence of *Bacillus subtilis* on such assimilation. Soil Sci. **3**: 155–195. 1917.
5. Clark, N. A. The rate of reproduction of *Lemna major* as a function of intensity and duration of light. Jour. Physiol. Chem. **29**: 935–941. 1925.
6. Clark, N. A. Technique for the growth of Lemna under sterile conditions with controlled temperature and light. Iowa State Coll. Jour. Sci. **7**: 13–16. 1932.
7. Fly, C. L. Organic ion and hydrogen ion concentration as associated factors affecting the rate of reproduction of *Lemna major*. Proc. Oklahoma Acad. Sci. **15**: 77–80. 1935.
8. Ghosh, B. P. and Burris, R. H. Utilization of nitrogenous compounds by plants. Soil Sci. **70**: 187–203. 1950.
9. Gile, P. L. and Carrero, J. O. Assimilation of iron by rice from certain nutrient solutions. Jour. Agr. Res. **7**: 503–528. 1916.
10. Guillon, J. M. Experiments in the treatment of chlorosis. Prog. Agr. et Vit. **12**: 653–655. 1895.

11. HOPKINS, E. F. and WANN, F. B. Relation of hydrogen-ion concentration to growth of Chlorella and to the availability of iron. Bot. Gaz. **81**: 358–376. 1926.
12. HOPKINS, E. F. and WANN, F. B. Iron requirement for Chlorella. Bot. Gaz. **84**: 407–427. 1927.
13. KLEIN, G. Changes in the nitrogen of green plants. Ergeb. Agr. Chem. **2**: 143. 1930.
14. LANFORD, O. E. and KIEHL, S. J. A study of the reaction of ferric ion with orthophosphate in acid solution with thiocyanate as an indicator for ferric ions. Jour. Amer. Chem. Soc. **64**: 291–296. 1942.
15. LANFORD, O. E. and QUINAN, J. R. A spectrophotometric study of the reaction of ferric iron and citric acid. Jour. Amer. Chem. Soc. **70**: 2900–2903. 1948.
16. OLSEN, C. Iron absorption and chlorosis in green plants. Compt. rend. trav. lab. Carlsberg, Ser. Chim. **21**: 15–52. 1935. (Chem. Abstracts **29**: 7398. 1935.)
17. PRIANISHNIKOV, D. N. Assimilation of ammonia by higher plants. Compt. rend. **177**: 603–606. 1923. (Chem. Abstracts **18**: 101. 1924.)
18. PRIANISHNIKOV, D. N. Ammonia in fertilizers and its relation to the life of plants. Trans. Sci. Inst. Fert. (Moscow) **61**: 99–103. 1929. (Chem. Abstracts **23**: 5264. 1929.)
19. PRIANISHNIKOV, D. N. The ammonia nutrition of higher plants. Biochem. Zeit. **207**: 341–349. 1929. (Chem. Abstracts **23**: 3489. 1929.)
20. SCHREINER, O. and REED, H. S. The toxic action of certain plant constituents. Bot. Gaz. **45**: 73–102. 1908.
21. SIELING, D. H. The influence of the phosphate–calcium ratio and of humates on chlorosis in Lemna. Iowa State Coll. Jour. Sci. **12**: 151–154. 1937.
22. SMITH, G. F. and RICHTER, F. P. Phenanthroline and substituted phenanthroline indicators. Pp. 67–77. G. Frederick Smith Chemical Co., Columbus, Ohio. 1944.
23. STEINBERG, R. A. Use of Lemna for nutrition studies on green plants. Jour. Agr. Res. **62**: 423–430. 1941.
24. STEINBERG, R. A. Use of Lemna as a test organism. Chronica Botanica **7**: 420. 1943.
25. STEINBERG, R. A. Mineral requirements of *Lemna minor*. Plant Physiol. **21**: 42–48. 1946.
26. STRUTHERS, P. H. and SIELING, D. H. Effect of organic anions on phosphate precipitation by iron and aluminum as influenced by pH. Soil Sci. **69**: 205–213. 1950.
27. WHITE, H. L. The interaction of factors in the growth of Lemna. VIII. The effect of nitrogen on growth and multiplication. Ann. Bot. **50**: 403–417. 1936.

IDENTIFICATION OF ETHYLENE AS A VOLATILE PRODUCT OF THE FUNGUS *PENICILLIUM DIGITATUM* [1]

ROY E. YOUNG, HARLAN K. PRATT AND J. B. BIALE

(WITH THREE FIGURES)

Received July 11, 1950

Introduction

This paper presents the chemical identification of ethylene as a metabolic product of the fungus *Penicillium digitatum* Sacc. It also presents a simplified method for the accumulation and identification of the small concentrations of ethylene which may be produced by plant materials.

ETHYLENE PRODUCTION BY FUNGI

Experiments reported independently by BIALE (**1**) and MILLER, WINSTON, and FISHER (**8**) revealed that the common green mold of citrus fruits, *P. digitatum*, produces a physiologically active emanation whose effects on plant tissue are the same as those caused by ethylene gas. It was shown by the latter authors and by BIALE and SHEPHERD (**3**) that this fungus produces this emanation whether grown on its natural host or in culture, and PRATT (**10**) showed that production of the emanation does not depend on major nutrient components of the culture solution but does appear to be related to active growth and respiration of the culture. Production of the active gas by fungus-infected fruit has been further studied by ROHRBAUGH (**12**) and BIALE (**2**).

Except for recent evidence of NICKERSON (**9**) that ethylene may be produced by *Blastomyces dermatitidis* and two other human pathogens, *P. digitatum* is the only fungus reported to have this activity. Biological testing of various other plant pathogens has given negative results (**1, 14**). Since *P. digitatum* is a particularly promising organism for the study of the mechanism of ethylene formation, it was of interest to verify chemically the identity of the active gas produced.

IDENTIFICATION OF ETHYLENE

The present work is based on the method of PRATT *et al.* (**11**), except that the accumulation of the ethylene and formation of ethylene dibromide have been greatly simplified. Instead of bromine, mercuric perchlorate has been used to absorb the ethylene from the air stream which has been passed over the plant tissue.

HOFMANN and SAND (**7**) found that solutions of mercuric salts take up

[1] A cooperative project of the Division of Subtropical Horticulture and the Division of Truck Crops, College of Agriculture, University of California, carried out in the laboratories of the former division.

olefins, forming complex molecules from which the olefin is released quantitatively upon addition of hydrochloric acid. HANSEN and HARTMAN (**6**) and DENNY (**4**) adapted these findings to the absorption of ethylene from an atmosphere, using mercuric nitrate solution to accumulate ethylene produced by plant tissue. They then added hydrochloric acid to the solution to free the ethylene in a small volume of air so that it could be identified by a biological test. The work of GROSS (**5**) suggested the use of mercuric perchlorate as the absorbent. Comparison with other mercuric salts in our laboratory showed the perchlorate to be best for the efficient absorption and subsequent quantitative release of ethylene. For the identification of ethylene produced by *Penicillium digitatum*, a solution of mercuric perchlorate was used to scrub this gas from a stream of air which was passing continuously over a series of cultures. The accumulated ethylene was subsequently released into a reaction flask by the addition of hydrochloric acid to the solution containing the ethylene mercury complex. The ethylene was brominated in the reaction flask, and suitable derivatives were produced from the ethylene dibromide.

Culture of the fungus

The strain of *Penicillium digitatum* used was collected in the field from a typically decaying grapefruit, as recommended by THOM (**13**, p. 244), and its purity was assured by five single-spore isolations. It was tested again on citrus fruit to verify its typical behavior. Several years' work with this fungus has shown little variability between isolates and strains with regard to gross morphology, behavior on fruit, and ability to produce ethylene (as shown by biological tests).

For this project, the fungus was grown as a surface mat on shallow layers of liquid medium, using the formula of PRATT (**10**) as follows (per liter of water): 25.7 gm. sucrose, 4.00 gm. NH_4NO_3, 13.61 gm. KH_2PO_4, 1.23 gm. $MgSO_4 \cdot 7\,H_2O$, 0.02 gm. $FeSO_4 \cdot 7\,H_2O$, 0.50 gm. Difco yeast extract, and 1.0 ml. of a micromineral supplement which provided in the final solution 0.02 p.p.m. of copper, 0.05 p.p.m. zinc, 0.01 p.p.m. molybdenum, 0.5 p.p.m. manganese, and 0.5 p.p.m. boron. The pH was adjusted to 5.0.

Thirty-eight Fernbach culture flasks were supplied with 200 ml. of culture solution each, capped with paper, and sterilized in the autoclave at 15 pounds for 15 minutes. Assemblies of rubber stoppers with glass input and outlet tubes and attached cotton-containing guard tubes (fig. 1, E . . . E′) were placed in paper bags and autoclaved separately. After sterilization, the flasks were inoculated with equal portions of a heavy spore suspension, and the sterilized stopper assemblies were inserted and tied down. The flasks were then assembled in series with cotton guard tubes between each flask, and air was passed through the series at approximately 350 ml. per minute. Throughout the experiment, the cultures and absorption train were maintained at 25° C. (Later work has shown that the absorber should be maintained in an ice bath for maximum efficiency.)

Accumulation and identification of ethylene

Absorption train

The apparatus used for the absorption of the active emanation of *Penicillium digitatum* is diagrammed in figure 1. Air was passed through a tower of brominated activated charcoal (Columbia 4ACW, size 6/14) to remove possible active contaminants. This tower consisted of a tube 50 mm. in diameter and 80 cm. long, filled one-third full (A) of brominated carbon (24 ml. of bromine on 200 gm. of carbon), followed by twice this volume of unbrominated carbon (B). The efficiency of this method of removal of possible contamination from the air stream has been verified previously (11).

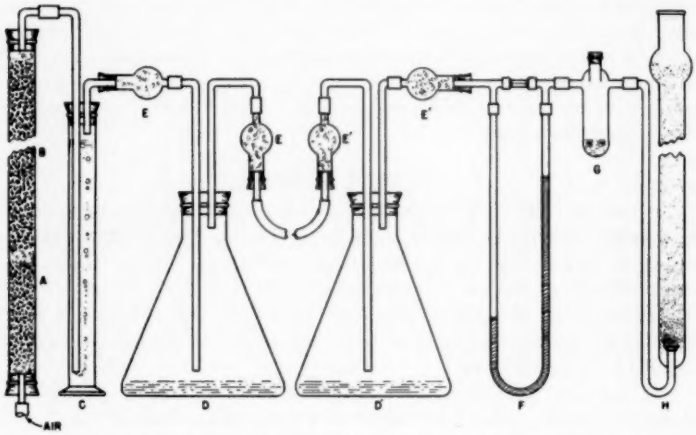


Fig. 1. Absorption train for collection of ethylene produced by fungus.

After purification, the air was humidified by a water tower (C) and passed over the fungus cultures contained in 38 Fernbach flasks (D . . . D') connected in series, through a capillary flowmeter (F), and then through a small bulb (G) containing *n*-butyl alcohol. The slow evaporation of the butyl alcohol into the air stream maintained the low concentration of alcohol in the absorber necessary to give optimum bubble size and efficiency. The absorber (H) consisted of a tube 25 mm. in diameter and 60 cm. long with a fritted Pyrex disc sealed in the inlet tube.

The absorbent solution was made up as follows: 50 gm. of mercuric oxide were dissolved in 177 ml. of 70% perchloric acid diluted to approximately 8 *N*. This solution was filtered by suction through an asbestos pad, and made to a final volume of one liter with water. The resulting solution was approximately 5% mercuric oxide in 2 *N* perchloric acid. For the fungus emanation series 100 ml. of solution were used in the absorber.

The quantitative production of ethylene by the cultures was followed by

withdrawing ½-ml. aliquots of the absorbent solution daily. From these the ethylene was released and measured manometrically by a method to be reported elsewhere (**15**). As shown by figure **2**, ethylene production dropped markedly on the 10th day after inoculation, and the experiment was then terminated.

A parallel series without the cultures was set up and operated as a control on the air stream and reagents during the course of the absorption. In this series, 25 ml. of solution were used in an absorber of the same type but

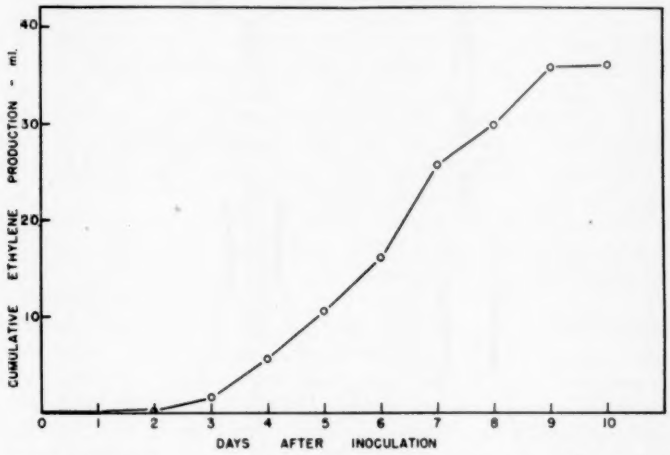


FIG. 2. Cumulative ethylene production of 38 cultures of *Penicillium digitatum*. (Final dry weight 34 gm.)

of proportionally smaller size. Absence of any absorbed gas in the control was established by use of the quantitative manometric determination mentioned above.

RELEASE AND BROMINATION OF ETHYLENE

The reaction assembly used for the release of ethylene from the mercury complex and for bromination of the ethylene is illustrated in figure 3. It consisted of two 250-ml. Erlenmeyer flasks with 24/40 standard taper joints, joined by a stopcock assembly for transferring the released gas from flask A to flask B.

The absorbent solution containing the ethylene-mercury complex was placed in flask A, and the system was evacuated to a pressure of 50 mm. of mercury through stopcock C, with E closed and F open. Stopcock C was then turned so as to close the system but leave flasks A and B connected, and a separatory funnel containing 6 *N* hydrochloric acid was connected to stopcock E. Approximately 100 ml. of the acid was admitted through E,

and the mixture was vigorously shaken. (The operator had to be alert to close stopcock F temporarily if too much foam developed.) The ethylene was released rapidly and passed into flask B. Additional small portions of the acid were added, with shaking, until no further evolution of the gas was observed, and then acid was allowed to run in and fill flask A to stopcock C, displacing all gas from the flask. Stopcocks C and F were then closed, and

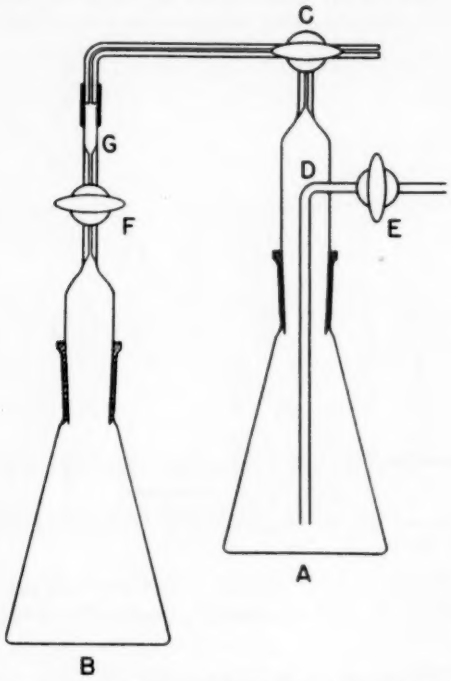


FIG. 3. Apparatus for release and bromination of ethylene.

the space between them was swept with several small portions of air by alternately opening C with F closed and then opening F with C closed. (It was important to retain a considerable vacuum in flask B to draw in subsequent reagents.)

With stopcock F closed, flask assembly B was disconnected, and a few drops of bromine were placed in the chamber at G and cautiously drawn in by opening the stopcock. Most of the bromine added evaporated, and the reaction took place rapidly, the dibromide condensing out on the vessel walls. The reaction flask was warmed gently over a steam bath to increase the reaction rate and encourage the product to flow down the walls of the

flask and collect at the bottom. After 30 minutes, small portions of sodium bisulphite solution were drawn into the flask until all free bromine disappeared. The reaction mixture was then extracted with ether, the first portion being drawn into the flask through the stopcock by the residual vacuum. After three more extractions, the ether solution was transferred to a 12-inch test tube. The ether was carefully evaporated over steam, leaving a small amount of yellow oil, presumably ethylene dibromide.

Preparation of derivatives

For final identification derivatives were prepared as previously reported (**11**). An excess of aniline was added, and the mixture was heated on the steam bath for three and one-half hours. The unreacted aniline was then removed by steam distillation, and the resulting aqueous mixture was extracted with chloroform. After separation the chloroform was evaporated off, leaving a dark brown residue, presumably N,N′-diphenylethylenediamine. This was recrystallized three times from 50% ethanol, and the resulting crystals melted at 66.5-67.0° C, as did a known sample (Eastman White Label) and a mixture of these. The melting points were uncorrected and were taken simultaneously on the aluminum block of a Fisher-Johns melting-point apparatus.

The above derivatives were converted to N,N′-diphenyl-N,N′-dianilinothioformylethylenediamine by reaction with phenylisothiocyanate, and the products were washed three times with Skellysolve B and three times with 95% ethanol, filtered with suction, and dried under vacuum for 36 hours over P_2O_5. Melting point data (taken as above): from *Penicillium digitatum* derivative 188.5–189.0° C; from N,N′-diphenylethylenediamine (Eastman White Label), 189.0–189.5° C; mixture of these, 189.0–189.5° C.

Summary

A greatly simplified method has been devised for the collection and identification of small concentrations of ethylene from gas streams, the principal feature being the concentration of the ethylene in a mercuric perchlorate solution with subsequent release and bromination under easily controlled conditions. The ethylene dibromide obtained was identified as N,N′-diphenylethylenediamine and N,N′-diphenyl-N,N′-dianilinothioformylethylenediamine. Using this technique, ethylene was identified as a volatile product of the common green mold of citrus fruits, *Penicillium digitatum* Sacc.

University of California
Division of Subtropical Horticulture
Los Angeles 24, California

Division of Truck Crops
Davis, California

LITERATURE CITED

1. Biale, J. B. Effect of emanations from several species of fungi on respiration and color development of citrus fruits. Science **91**: 458–459. 1940.
2. Biale, J. B. Respiration of citrus fruits in relation to metabolism of fungi. II. Effects of emanations of Penicillium digitatum Sacc. on lemons at different stages of ripeness. Proc. Amer. Soc. Hort. Sci. **52**: 187–191. 1948.
3. Biale, J. B. and Shepherd, A. D. Respiration of citrus fruits in relation to metabolism of fungi. I. Effects of emanations of Penicillium digitatum Sacc. on lemons. Amer. Jour. Bot. **28**: 263–270. 1941.
4. Denny, F. E. Leaf-epinasty tests with volatile products from seedlings. Contrib. Boyce Thompson Inst. **9**: 431–438. 1938.
5. Gross, C. R. Determination of ethylene with simultaneous estimation of total oxidizable volatiles other than ethylene. New York (Cornell) Agric. Exp. Sta. Dept. Pomology, Mimeograph. 1947.
6. Hansen, Elmer and Hartman, Henry. The occurrence in pears of metabolic gases other than carbon dioxide. Oregon Agric. Exp. Sta. Bul. 342. 1935.
7. Hofmann, K. A. and Sand, Julius. Ueber das Verhalten von Mercurisalzen gegen Olefine. Ber. **33**: 1340–1353. 1900.
8. Miller, Erston V., Winston, J. R., and Fisher, D. F. Production of epinasty by emanations from normal and decaying citrus fruits and from Penicillium digitatum. Jour. Agric. Res. **60**: 269–277. 1940.
9. Nickerson, Walter J. Ethylene as a metabolic product of the pathogenic fungus, Blastomyces dermatitidis. Arch. Biochem. **17**: 225–233. 1948.
10. Pratt, Harlan K. Studies on the physiology of Penicillium digitatum Saccardo. Doctoral Dissertation, University of California, Los Angeles. 1944. (Abstract in Amer. Jour. Bot. **31**(8): 11s. 1944.)
11. Pratt, Harlan K., Young, Roy E., and Biale, J. B. The identification of ethylene as a volatile product of ripening avocados. Plant Physiol. **23**: 526–531. 1948.
12. Rohrbaugh, P. W. Measurement of small concentrations of ethylene and automobile exhaust gases and their relation to lemon storage. Plant Physiol. **18**: 79–89. 1943.
13. Thom, Charles. The Penicillia. Williams and Wilkins Co. Baltimore. 1930.
14. Williamson, C. E. Ethylene, a metabolic product of diseased or injured plants. Phytopath. **40**: 205–208. 1950.
15. Young, Roy E., Pratt, Harlan K., and Biale, J. B. Micro-manometric estimation of ethylene in the emanations of ripening fruit. In preparation. (Abstract in Amer. Jour. Bot. **35**: 814. 1948.)

STUDIES ON THE PHYSIOLOGY OF LIGHT ACTION. IV. LIGHT ENHANCEMENT OF AUXIN-INDUCED GROWTH IN GREEN PEAS

ARTHUR W. GALSTON AND ROSAMOND S. BAKER

(WITH SEVEN FIGURES)

Received April 22, 1950

In the first paper of this series (**3**), it was demonstrated that the auxin-induced growth in length of sub-apical sections of etiolated pea stems is greatly inhibited by light. This light-growth inhibition was subsequently (**1, 2**) interpreted in terms of a riboflavin-sensitized photoinactivation of indoleacetic acid (IAA). Such an interpretation derives support from the fact that light, concurrent with its inhibition of growth, increases the rate of disappearance of IAA from the external medium.

In the course of similar experiments with stem sections of light-grown peas, we noticed that light enhances, rather than inhibits, the auxin-induced growth. This response was unexpected and of such great magnitude that it was considered desirable to investigate it more fully. The present paper describes the phenomenon in some detail and presents an explanation of it which seems consistent with all the facts at our disposal. The simple section growth test herein described may, incidentally, be of some use to those interested in utilizing green tissues for auxin or herbicide studies.

Materials and methods

Alaska peas were thickly sown in coarse gravel in plastic boxes 4 by 4 by 3 inches, and were watered twice daily with half-strength Hoagland's solution. In order to permit drainage of the excess nutrient solution from the box, several small holes were drilled in the bottom. The boxes were exposed, in the "phytotron" (**4**), to the following constant environmental conditions: temperature, 20° C; light, eight hours per day at 800 foot-candles of mixed "Daylight" and "White" fluorescent light augmented by incandescent bulbs. Under these carefully controlled conditions in the phytotron, the growth of the plants is extremely regular and reproducible. At the age of 12 days, the plants had five nodes visible on the stem. The subterminal portions of the stem (designated "A" on figure 1) were then utilized for section growth tests. In the selection of plants to be used in these tests, care was taken to reject any in which the sixth internode had started to elongate. In such plants, active growth is largely restricted to this sixth internode, and sections excised from the fifth internode will not grow rapidly. Sections of young petioles and tendrils may also be used in growth tests but are not as convenient for this purpose as the stem sections.

After the stems had been severed at the ground level, the expanded fifth

leaf and apical bud were cut off. The stems were then inserted into the "coleoptile microtome" previously described (**3**) and two successive sections, each 5.34 millimeters long, cut from the apical region. The cut sections were placed in a Petri dish containing a shallow layer of distilled water, and randomization in the tests insured by thorough mixing of the sections prior to their insertion into any experimental flask. In the tests, 18 sections were floated on the surface of 5 ml. of nutrient medium contained in a 50-ml. Erlenmeyer flask. For the "dark" treatment, the flasks were kept in an absolutely dark growth chamber; for the light treatment, they were exposed to about 400 fc of mixed "Daylight" and "White" fluorescent illumination. The temperature was maintained at about 25° C in both growth chambers.

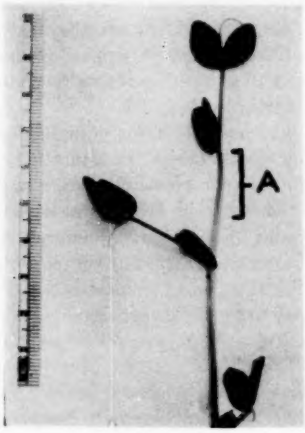


FIG. 1

FIG. 2

FIG. 1. A typical 12-day-old plant utilized in the section growth experiments. "A" designates sub-apical region from which the sections were cut.

FIG. 2. The interaction of light and auxin in the growth of green pea stem sections. Left: light, no auxin; center: auxin and light; right: auxin, no light.

The sections were harvested after 16 to 24 hours, lined up on a microscope slide, and their length measured to the nearest 10th of a millimeter with an ocular micrometer. Group averages and standard errors were then computed.

The appearance of the sections and their variability within any one group may be seen from figure 2. In general, growth is not as uniform in this test as in the test involving etiolated sections. In addition, green sections sometimes exhibit contorted growth, making straight-growth measurements difficult. Despite these limitations, the test gives statistically valid data because of the great elongation accomplished by the sections under favorable conditions.

Experimental

Response of green sections to auxin

Previous experiments with etiolated pea sections had shown that good growth could be obtained in a medium containing 2% sucrose, M/60 pH 6.1 phosphate buffer and 0.1–1.0 mg./l. IAA. In the preliminary tests on the growth of green sections, similar media were employed, with the IAA being varied over a wide concentration range, from 0 to 100 mg./l. Representative results from such growth tests are shown in figures 2 and 3. It is

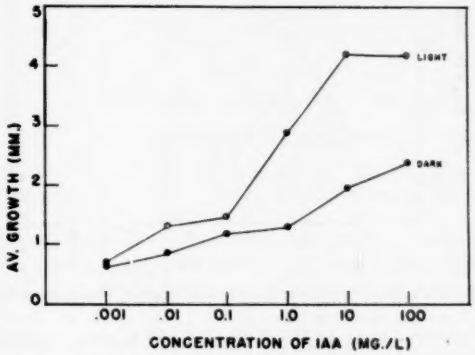


Fig. 3. The effect of auxin concentration on growth of green pea stem sections in light and dark.

apparent that practically no elongation occurs in the absence of IAA. In the presence of IAA in the dark, there is somewhat greater elongation, but when both light and IAA are supplied, the sections may elongate 80% or more in 16 hours. The optimum IAA concentration at this level of sucrose appears to be in the range 10–30 mg./l., a figure at least 100× higher than

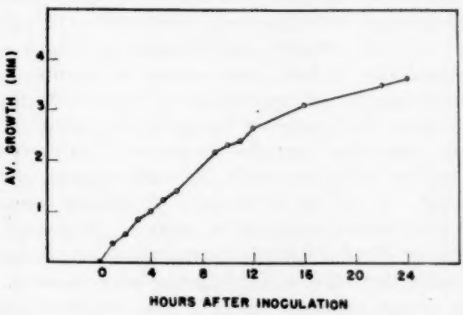


Fig. 4. The time course of growth of green pea stem sections.

the optimal IAA level for etiolated sections. The time course of growth of sections exposed to 10 mg./l. IAA in the light is shown in figure 4. Unlike the etiolated sections, which had practically completed their growth at the end of 12 hours, the green sections continue their growth up to (and presumably beyond) the 24-hour point on the curve. The effect of the pH of the medium on the growth of sections is shown in figure 5. A rather broad

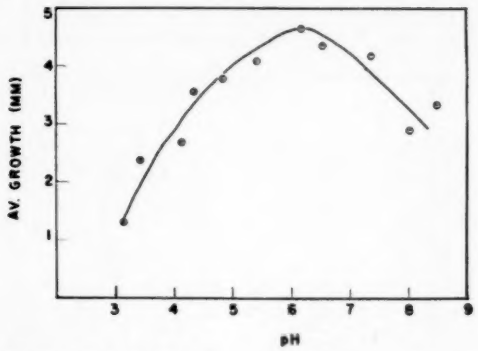


FIG. 5. The effect of pH on the growth of green pea stem sections.

optimum for growth exists in the region of pH 6, thus justifying the continued used of M/60 pH 6.1 KH_2PO_4–Na_2HPO_4 buffer.

THE NATURE OF THE LIGHT EFFECT

It will be recalled that periods of illumination as short as one minute or less are effective in causing marked inhibition of growth of etiolated sections (**3**). Obviously, the photochemical reactions involved in such a growth inhibition are consummated by small quantities of light energy. For the sake of convenience, we can refer to such an effect as "microquantic," in contrast with photo-reactions like photosynthesis which require much higher amounts of light energy, and which may therefore be referred to as "macroquantic." Our next experiments were designed to discover whether the light-growth stimulation of these green sections in the presence of 10 mg./l. of IAA is a "microquantic" or "macroquantic" type of effect.

Sections were cut and placed in the dark into media lacking sucrose. Individual flasks were then permitted to receive from three seconds to 24 hours of illumination, being removed to the dark chamber at the conclusion of the light period. At the end of 24 hours, all sections were harvested and measured. Typical results are shown in figure 6. In general, it was found that the stimulatory effect of light on growth increases with increasing exposure time, no saturation effect of light having been observed. This differs from the light dosage relations observed with etiolated tissues (**3**) and probably justifies our calling this a "macroquantic" type of effect.

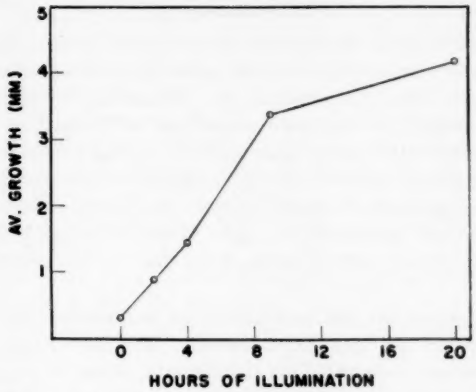


FIG. 6. The effect of duration of illumination on the growth of green pea stem sections provided with auxin. Light intensity was approximately 400 fc.

In subsequent experiments, it was found that the addition of sucrose to the medium compensates in part for the action of light. This suggests that the "macroquantic" effect of light on growth is exerted through photosynthesis. Typical results (fig. 7) show that sections grown in the light are not benefited by the addition of sucrose, being in fact inhibited by concentrations in excess of 1%. Sections grown in the dark, on the contrary, are stimulated by sucrose, the maximum growth being attained at a level of 4%. Despite the addition of sucrose, the best growth attained in the dark is not as good as that attained in the light. This indicates that most, but not all of the light stimulation of growth may be interpreted in terms of a photosynthetic production of substrates essential for respiration and growth.

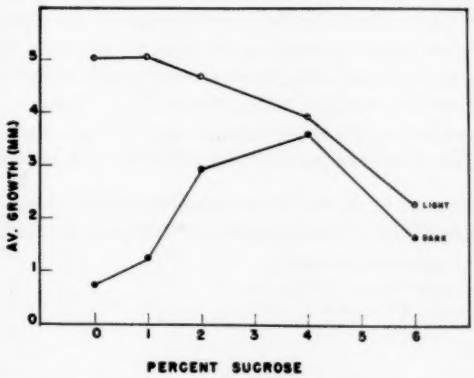


FIG. 7. The effect of sucrose concentration on the growth of green pea stem sections in light and dark.

Discussion

A comparison of the results obtained in growth studies on etiolated (**3**) and green pea stem sections reveals some interesting differences. The growth of stem sections of etiolated peas is inhibited by light, this effect being most probably due to auxin destruction in the medium. Green pea stem sections will also destroy more auxin in the light than they do in the dark, yet the overall effect of light is to stimulate their growth. The data of the present paper indicate that this effect can best be interpreted in terms of a photosynthetic production of sugar. Therefore, sugar is paradoxically more limiting to the growth of green tissue than to corresponding etiolated tissue.

It is also noteworthy that dark-grown pea sections are much more sensitive to auxin than are light-grown sections. The former have an optimal IAA concentration of about 0.1 mg./l.; the latter about 10 mg./l. This difference can probably be attributed to auxin photoinactivation in the green plant. The fact that the addition of auxin increases the growth of green sections raises the problem of why application of auxin to intact green plants does not similarly stimulate growth. This and related problems are under investigation.

Summary

1. Excised stem sections of green pea plants will grow rapidly in fairly intense light if they are provided with 10–30 mg./l. of indoleacetic acid. Even at this optimal concentration of auxin, the sections grow poorly if kept in the dark. The light effect is probably due to photosynthesis, since illumination can be replaced by 4% sucrose.

2. The differences in behavior between stem sections of etiolated and green peas may be summarized as follows: Light inhibits the growth of etiolated pea epicotyl sections by inactivating auxin, which limits the growth of this tissue; light stimulates the growth of green pea stem sections by causing a photosynthetic production of sugar, which limits growth in this tissue. Thus, these green tissues are apparently more limited by sugar than are the corresponding etiolated non-photosynthetic tissues.

3. The green pea section growth test is suggested as a convenient screening procedure for testing the growth-regulatory effects of various compounds.

The authors wish to express their appreciation to Professor F. W. Went for the use of facilities in the phytotron, and for several useful suggestions.

KERCKHOFF LABORATORIES OF BIOLOGY
CALIFORNIA INSTITUTE OF TECHNOLOGY
PASADENA 4, CALIFORNIA

LITERATURE CITED

1. GALSTON, A. W. and BAKER, R. S. Studies on the physiology of light action. II. The photodynamic action of riboflavin. Amer. Jour. Bot. **36**: 773–780. 1949.
2. GALSTON, A. W. and BAKER, R. S. Studies on the physiology of light action. III. Light activation of a flavoprotein enzyme by reversal of a naturally occurring inhibition. Amer. Jour. Bot. (In press).
3. GALSTON, A. W. and HAND, M. E. Studies on the physiology of light action. I. Auxin and the light inhibition of growth. Amer. Jour. Bot. **36**: 85–94. 1949.
4. WENT, F. W. The Earhart Plant Research Laboratory. Chronica Botanica **12**: Reprint No. 2. 1949.

PHOTOTUBE-TYPE INTEGRATING LIGHT RECORDERS: A SUMMARY OF PERFORMANCE OVER A FIVE-YEAR PERIOD

G. Fred Somers and Karl C. Hamner[1]

(WITH EIGHT FIGURES)

Received June 28, 1950

In recent years considerable attention has been given to a study of the relationship between the amount of light a plant receives and its vitamin content (**1, 2,** and **3**). Such a relationship can be studied under laboratory and greenhouse conditions, but ultimately it has to be evaluated under field conditions. This requires some measure of the amount of sunlight. Various instruments are available for recording the amount of sunlight. One, used by the Weather Bureau, is the Eppley pyrheliometer, which is a multiple-junction thermocouple combined with a millivolt recorder. While this instrument gives a continuous and reasonably dependable record of the amount of solar radiation, it has certain drawbacks which limit its use under field conditions. It is expensive and requires an external source of electricity to operate the recorder. The record obtained must be integrated in some fashion, which either requires additional time in making use of the data obtained or requires the purchase of a rather expensive integrating unit. Furthermore, the light receiver is a horizontal disc, whereas in certain cases an opal glass globe receiver would appear to be more desirable (**7**). An instrument which appears to be more suitable for field experiments was described by Sprague and Williams (**5, 6**). This instrument is simple to construct, is portable and is battery operated. It uses an opal glass globe receiver of the type Wallace (**7**) found to be desirable for use in plant physiological experiments.

Some years ago we were confronted with the problem of measuring sunlight under field conditions. After considering various possibilities we decided to construct a number of light recorders similar to the one described by Sprague and Williams (**6**). In constructing these instruments we had several aims: (1) To obtain dependable daily values for the amount of sunlight with a minimum of effort, (2) To be able to obtain these records under field conditions without being dependent upon an external source of electricity, (3) To obtain instruments which were readily portable and could be shipped readily to various cooperators.

Details of construction

The circuit which was adopted after making various preliminary tests is shown in figure 1. This differs in several details from the instrument

[1] Present address: Department of Botany, University of California, Los Angeles, California.

described by SPRAGUE and WILLIAMS (**6**), but the principle of operation is essentially the same. It will be noted that eight counters were provided. By means of a commutator these give a record for the total amount of light per day for a period of a week. The counters can be read any time on the eighth day, at which time the commutator is set for another week. The power for the counters is supplied either by 110-volt AC or from a 6-volt DC source (four 1½-volt No. 6 Eveready "Ignitor" batteries). The ratings for other components of the circuit are as follows: Condensers: C_1, 0.02 μF; C_2, 9 to 12 μF; Resistors: R_1, 50,000 ohms; R_2, 250,000 ohms; R_3, depends upon the relay. The sum of relay-coil resistance and R_3 was 2500 to 3500 ohms. A sensitive relay with a 6-volt coil and 115-volt AC, 6-ampere contacts (No. 29XAX, Struthers Dunn, Inc., Philadelphia) is satisfactory for the recorders which use 115 volts AC to operate the counters. For the

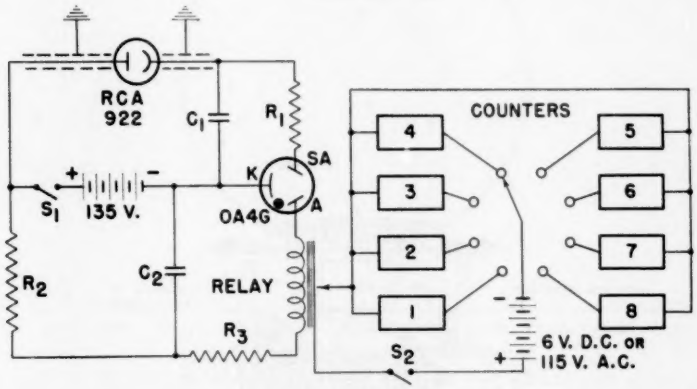


FIG. 1. Diagram of circuit used in phototube-type light recorders. RCA922 is a vacuum type phototube. OA4G is a cold cathode relay tube.

battery-operated units a more sensitive relay with a 3500-ohm coil is more satisfactory (Series 5 DC, with S.P.D.T. #1 contacts, Guardian Electric Co.). The 135-volt battery is composed of three Eveready No. 482 batteries or equivalent. The counters are Mercury (Production Instrument Company, Chicago, Illinois) five-digit, non-reset counters with either 6-volt DC or 115-volt AC coils, depending upon the power supply.

The phototube is housed in a brass case (see fig. 2) on which an opal glass globe such as is used in light fixtures is mounted with a vapor-tight seal of caulking compound (not shown in the figure). A brass sleeve extends into the throat of the globe, and in this are mounted two glass filters (Corning Nos. 2962 and 3850) and a ground-glass plate. These are held in a special support which has a one-inch aperture. On top of the glass filters is an aluminum disk (not shown in the figure) in which were punched holes (3/32 inch diameter) at regular intervals. The number of holes varies

some from recorder to recorder. The number is varied to give the desired response, but usually six are used. The phototube housing is kept dry by means of a container of anhydrous calcium sulphate (indicating Drierite). Originally the phototube was connected by means of a shielded weatherproof antenna cable to an aluminum box in which were housed C_1, C_2, R_1, the OA4G relay tube, and, initially, the relay. This box was also kept dry with calcium sulphate. Later the relays on the DC units were mounted externally so they could be serviced readily. Also, it was found desirable to use two, separate, shielded, heavily insulated cables to connect the photo-

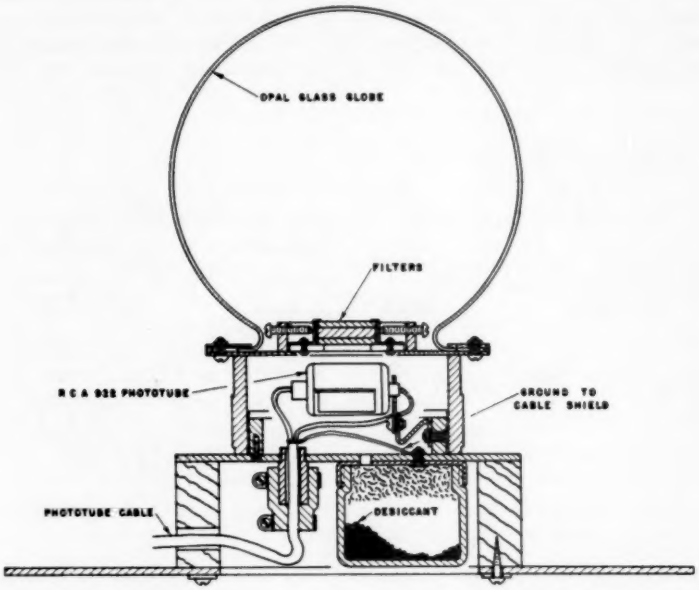


FIG. 2. Vertical cross section of phototube housing as originally built. In later models two phototube cables were used.

tube to the recorder. An even better arrangement apparently would be to include C_1 and the relay tube OA4G in the same housing with the phototube.[2] The commutator for the DC-operated units is coupled through an appropriate set of gears with the clock drum of a hygrothermograph and makes one revolution in eight days. This commutator and clock are synchronized each week when the counters are read.

The construction of the commutators used on those recorders which

[2] Personal communications from Radio Frequency Laboratory, Boonton, New Jersey, and W. H. Brittingham, Texas Agricultural Experiment Station.

required AC power supply is essentially the same, except that in this case the commutator is driven through a friction clutch by an AC clock motor. The rotor of this commutator also is driven at the rate of one revolution each eight days, and its position is indicated by an 8-day dial. Since the drive is by means of a friction clutch, the setting of the commutator can be changed readily at any time. The usual practice is to set the commutator to the correct time for Day No. 1 on Monday of each week at which time the counters are read.

The recorders using a DC power supply can be placed in weather instrument cabinets in the field adjacent to the experimental plots. Those using an AC power supply must be near a source of electric current and are ordinarily placed in a building with the phototube housing outside. Both types

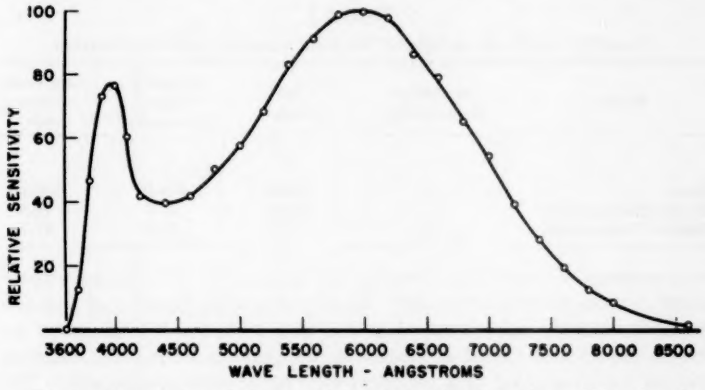


FIG. 3. Approximate relative sensitivity of the 922 phototube to light of various wavelengths when combined with the filter combination used.

of recorder have been shipped by express for long distances at various times with little or no damage.

The transmission of the filter combination used in the phototube housing was measured with a Beckman spectrophotometer. These data were combined with data supplied by the Radio Corporation of America on the spectral sensitivity of the phototube to obtain a curve representing the spectral sensitivity of the RCA 922 Phototube in combination with these filters (see fig. 3). Due to the variations in filters, phototubes, and opal glass globes, this curve is probably only an approximation of the spectral sensitivity of any one instrument. The phototube alone has a sensitivity response with peaks of relatively high sensitivity at about 3600 and 8000 Å. These peaks are eliminated by the filters and the response is limited essentially to the visible region of the spectrum. Our measurements indicate that the opal glass globe influences this relative spectral sensitivity little, if any.

Performance

The criteria used to evaluate these instruments were: (1) linearity of response, (2) sensitivity to light of low intensities, (3) ruggedness, (4) stability of the calibration between recorders, (5) performance and durability of mechanical components and (6) comparison with the Eppley pyrheliometer over extended periods of time. This comparison was made because the pyrheliometer is an accepted standard for measuring solar radiation.

Linearity of response was checked in two ways. A limited test was made by shading the receiver with single or double thicknesses of cheesecloth, timing the counting rate, and measuring the light intensity with a Weston Sunlight Illumination Meter (Model No. 603). The observations are summarized in table I. In all cases the light intensities were the maximum readings obtainable by directing the target of the Weston light meter toward various portions of the sky. To further test the linearity of response the light intensity was measured in the open with the Weston meter on several days which varied in cloudiness, and simultaneously the counting rates of the integrating light recorders were timed with a stopwatch. The performance was considered satisfactory in this respect when a linear response to the light intensity measured by the Weston meter was obtained. In some cases, minor adjustments had to be made to obtain the desired response. A typical response curve is shown in figure 4.

TABLE I

COUNTING RATE OF RECORDER NO. 3 AT VARIOUS LIGHT INTENSITIES

Shade	Number of observations	Light intensity	Relative light intensity	Relative counting rate
		(fc)		
None	4	6190	100.0	100.0
Single Cheesecloth	3	3230	52.2	50.8
Double Cheesecloth	2	1700	27.5	27.7

The sensitivity to light of low intensities was determined by measuring the light intensity (by Weston meter) at which the recorders stopped recording in the evening. This proved to be about 100 fc when measured in July, 1945, and a similar value was obtained in October, 1946.

The ruggedness of these recorders is indicated by the fact that during five years various recorders have been shipped by express to various parts of the United States and have been transported repeatedly over shorter distances by truck and car. In only one instance has a receiver been broken. In one or two other cases minor damage occurred, but the results demonstrate clearly that the instrument can be transported easily and without serious damage.

These recorders were used in field experiments by allowing them to operate simultaneously at our laboratory for a period, during which time the

relative number of counts per day was obtained for each instrument. The recorders were then sent into the field, one to each location. After the experiments were terminated, the recorders were returned to our laboratory, where they were again operated simultaneously to obtain their relative counting rates. When comparisons of this kind were made repeatedly over a period of years they provided some measure of the relative stability of

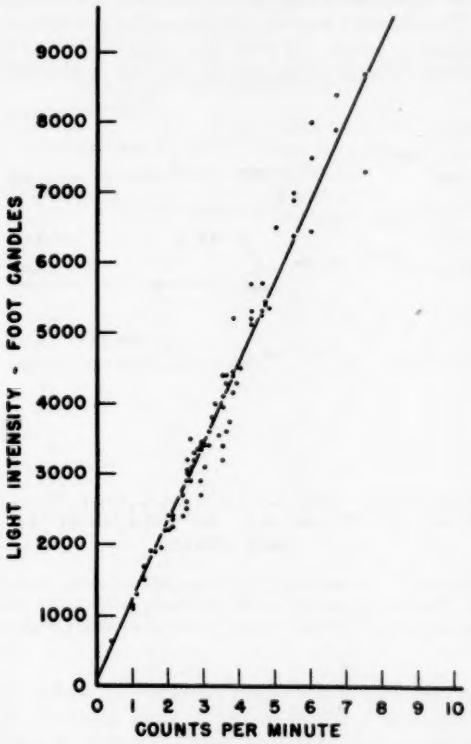


FIG. 4. Calibration of phototube-type light recorder with Weston light meter after installing dual phototube cables.

the different recorders. Some comparisons of this kind, obtained during three summers of operation, are summarized in figure 5. The periods of observation are indicated in each case by a heavy bar. The length of each bar indicates the length of the period used for the comparison. The data are typical of the results obtained with all of the recorders. They show that, while there is some variation among the recorders from time to time, they give reasonably reliable data. The variation in the relative counting

rate from time to time is apparently less than ± 5% in all cases, and considerably less than this in most cases. This is sufficiently accurate for most field experiments at the present time.

The only significant mechanical weakness encountered in these light recorders is the failure of the counters. The counters were inexpensive units (about $6.00 each) but the performance obtained was poorer than had been expected. Occasionally a counter will stick. This causes an erroneous record. This difficulty has been minimized by providing a ninth counter which is arranged to count each time any of the other eight counters registers. In this way, a total is obtained directly for each week. This can be

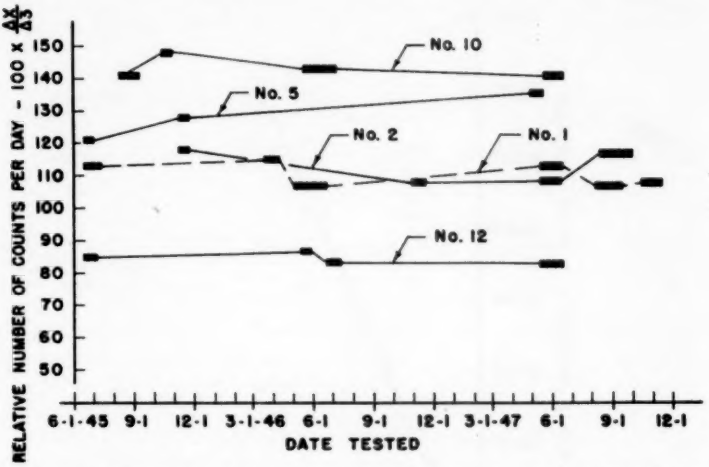


FIG. 5. A summary of relative response of six light recorders during various periods of observation. The duration of the periods of observation are indicated by the heavy bars. The response of each recorder (= Δx) is compared with the response of recorder no. 3 (= Δ3).

compared with the sum of the counts for each day during the week. Any discrepancy indicates a failure in one or more counters. Table II summarizes some comparisons between the total counts of the ninth counter and the sum of the other eight. It is seen that for the most part errors due to counter failure are small, but too frequently they are of serious magnitude. Counters which should prove to be more dependable are now available.

Some attempt has been made to obtain a comparison between these light recorders and an Eppley pyrheliometer installed on the Plant Science Building of Cornell University, which is a few hundred yards from our laboratory. Comparisons made between the pyrheliometer and the phototube-type recorders, remodelled with heavily insulated, separate phototube cables, during the years of 1947, 1948, and 1949 are summarized in figure 6. These data indicate that over this period of time the two types of instruments had

a rather constant relationship in their responses. There is no appreciable change from year to year. The correlation coefficient between the two sets of observations is $+0.968$ ($n = 285$).

Some further conclusions can be drawn from these data. It is obvious that there is some day-to-day variation in the response of the two types of instruments. Part of the observed variation may be due to errors such as the failure of counters, or errors in obtaining the data from the instruments.

TABLE II

PER CENT. DIFFERENCE BETWEEN SUM FOR THE INDIVIDUAL COUNTERS AND THE TOTAL OF A NINTH COUNTER.

i.e. $\frac{(\Sigma\Delta 1 + \Delta 2 \ldots\ldots\ldots \Delta 8) - \Delta 9}{\Delta 9} \times 100$, **WHERE** Δ **INDICATES THE NUMBER OF COUNTS**

Week ending (1948)		Recorder 1	Recorder 2	Recorder 5
May	31	...	+ 3.3	...
June	7	...	+ 1.7	...
	14	...	+ 0.60	+ 0.92
	21	...	− 1.3	0.00
	28	...	+ 0.46	...
July	5	...	− 1.7	+ 1.7
	12	...	− 1.19	− 0.025
	19	...	+ 0.014	+ 0.01
	26	+ 2.58	− 0.87	+ 3.7
August	2	+ 2.6	+ 16.3	+ 0.12
	9	...	...	+ 0.68
October	11	+ 0.050	− 0.12	...
	18	+ 4.04	− 10.6	...
	25	− 0.014	− 0.018	− 16.13
November	1	+ 0.017	− 0.007	− 4.84
	8	+ 9.75	− 2.12	− 4.44
	15	− 0.054	− 6.7	+ 6.95
	22	− 0.012	− 8.1	...
	29	− 0.18	0.00	...
December	6	− 0.012	0.00	...
	13	− 0.027	− 0.93	...
	20	− 0.19	− 4.50	...

However, part of the variation observed may result from real differences in the response of the two types of instruments. For example, since the pyrheliometer uses a horizontal disc receiver and the phototube-type recorder uses an opal globe receiver they may respond differently to diffuse light from the sky. Also, the disc-type receiver will be more sensitive to the declination of the sun than will the globe-type receiver. A linear regression line is drawn in figure 6. While this fits the data rather well it is probable that the relationship is not strictly linear. This is particularly true at the lower light values. Many of these latter values were obtained late in the year when the sun was at a relatively low angle above the horizon. It seems probable that this may account for the observed curvature.

An attempt was made to test this possibility. The values obtained from the Eppley pyrheliometer were adjusted for the expected effect of the time of the year upon the response of the instrument. A comparison of these adjusted values with the responses of phototube-type recorders is summarized in figure 7. This adjustment has resulted in somewhat more scatter-

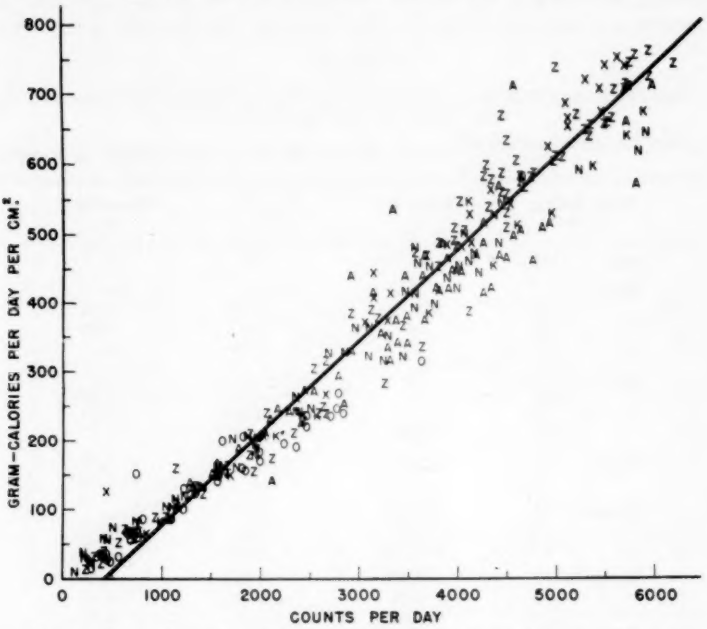


FIG. 6. Comparison of total daily radiation (gm. cal. per day per cm.²) as measured by an Eppley pyrheliometer with the total visible radiation measured by phototube-type light recorders (counts per day). The solid line is a regression line. The letters indicate observations made with various recorders at different times as follows: A, recorder no. 11, summer, 1948; K, recorder no. 5, spring, 1949; N, recorder no. 5, fall, 1947; O, recorder no. 2, fall, 1948; T, recorder no. 1, summer, 1947; X, recorder no. 12, summer, 1948; and Z, recorder no. 3, summer, 1948. All values for the phototube recorders are adjusted to a comparable basis.

ing of the points at the lower light values. The regression of the number of counts per day on the unadjusted daily values for the pyrheliometer (fig. 6) is:

$$\text{Counts} = 7.46 \text{ gm. cal./cm.}^2 + 428.$$

This indicates that the phototube-type recorder would give about 400 counts daily in the absence of any radiant energy measurable by the pyrheliometer. Repeated observations show that this is not the case. On the other hand the regression of the counts per day on the adjusted daily values for the pyrheliometer (fig. 7) is:

$$\text{Counts} = 4.60 \text{ gm. cal./cm.}^2 - 92.$$

This relationship fits observations on the response of the phototube-type recorder at low intensities much better. Moreover it agrees with the expected response of the two types of instruments as influenced by the times of the year. The globe type of receiver would be expected to be somewhat

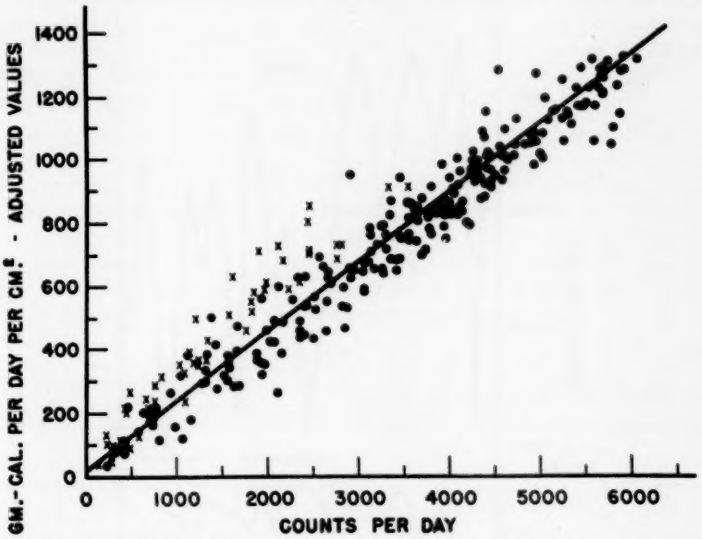


FIG. 7. Total daily radiation measured by an Eppley pyrheliometer and adjusted for the expected influence of the time of year upon the response, compared with counts per day obtained with phototube-type light recorder. The regression of counts per day upon the adjusted response of the pyrheliometer, using all the observations, is indicated by the line. ● represents values obtained in late spring and summer. X represents data obtained from October to December.

less sensitive to the angle of the sun (7) and the lower light values were obtained principally late in the summer and early fall.

When the response of the phototube-type recorder was compared with the adjusted values for the pyrheliometer it was observed that the relationship between the two instruments during the fall months was different from that obtained in the late spring and summer (fig. 7). The two regressions are:

April to September: Counts per day = 4.461 gm. cal. per day/cm.^{2} + 117
October to December: Counts per day = 3.459 gm. cal. per day/cm.^{2} − 68

A difference in response between the two instruments at different times of the year probably is to be expected. The receiver on the pyrheliometer is a horizontal disc which measures the total radiation received per unit of area tangential to the earth's surface. As a consequence this instrument

is rather sensitive to the angle between the receiving surface and the source of radiation. On the other hand, the phototube-type instrument with an opal globe receiver and light filters, as described above, gives an integrated

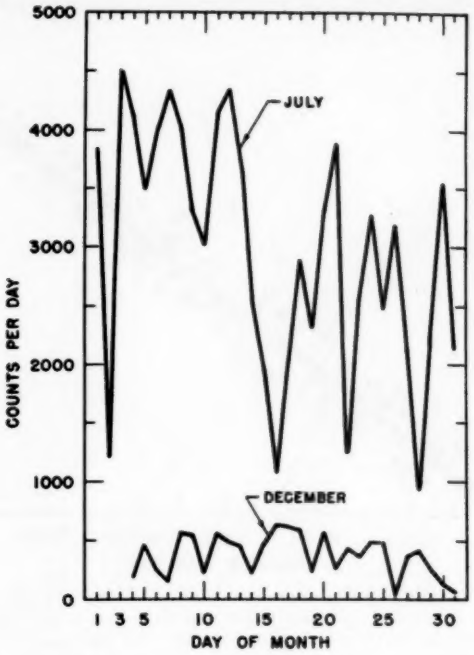


FIG. 8. A comparison of the total amount of visible radiation daily in the open during July and in the greenhouse in December. Both series of observations were made in Ithaca, New York, and are adjusted for differences in the counting rate of the recorders used.

measure of the total amount of visible radiation received by such a body. With such an arrangement, the angle between the receiver and the radiating source is of little or no consequence. Hence the two instruments may respond somewhat differently to reflected light from the sky, and the amount of such light probably varies with the season of the year. Ordinarily there is considerable cloudiness in Ithaca during the fall months. In addition, the two instruments would be expected to respond differently to changes in light quality, which will be influenced *inter alia* by the amount of moisture in the atmosphere. In view of such variables as these it is not surprising that there are differences in response between the two types of instruments. The day-to-day variation may be rather large, as is indicated by figures 6 and 7, but over a long period of time the correlation between the total radiation as measured by the pyrheliometer and the visible radiation as meas-

ured by the phototube-type of instrument is good. Too much significance should not be attached to individual daily variations since they may arise in part from mechanical errors in the instruments, particularly the failure of the counters used on the phototube-type of instrument. One of the most important conclusions to be drawn from this comparison between the two types of instruments is that the phototube-type recorder gave values which correlated closely with those given by the pyrheliometer over a period of years. The latter instrument has a reputation of dependability and this comparison speaks well for the phototube-type of instrument.

These recorders have provided a number of interesting comparisons of the relative amount of light under various conditions. A comparison of the relative illumination obtained at various locations in the United States during the summer of 1945 has been published (**4**). A comparison of the relative amount of light in various locations in one compartment of a small greenhouse for a period of about three weeks in May, 1946, is shown in table III. Data comparing the illumination outside in the summer with that in the greenhouse in the winter are summarized in figure 8.

TABLE III

ILLUMINATION, RELATIVE TO FULL SUNLIGHT, IN THREE LOCATIONS OF ONE COMPARTMENT OF A SMALL GREENHOUSE.

Location	Illumination relative to full sunlight
	%
Center of north bench	46.8
Center of east bench	32.7
Center of west bench	34.0

Discussion

These observations indicate that while the phototube-type integrating light recorder has certain drawbacks, it gives useful data and is reasonably dependable. The principal drawback in our present recorders is the occasional failure of the counters. Some investigation has been made of various possibilities for alleviating this difficulty. Two approaches now appear to be feasible. (1) A counter manufactured by Western Electric Company (Type 5S or 5AA message register) appears to be constructed in such a way that it would give more dependable service. (2) Most of our field experiments have indicated that daily values for the amount of light are really not necessary. If this is the case then the eight or nine counters used at present could be replaced with a single, more dependable counter. Such counters are available. Their use would simplify the construction of the recorders and would reduce their cost appreciably. Probably it is safe to say that, in view of our past experience, rather dependable and satisfactory light recorders of the phototube-type could be built easily and without

great expense. For some purposes a rather inexpensive unit could be designed.

Summary

A number of integrating light recorders have been built using a circuit similar to that described by SPRAGUE and WILLIAMS (**6**). When properly adjusted and constructed these give a linear response to sunlight at intensities greater than about 100 fc. These instruments have proved to be rugged and satisfactory in many respects. They are portable, and the battery-operated models can be placed directly in a weather instrument cabinet in the field where, if they are read and set once a week, they give an integrated value for the daily amount of light. These readings are closely correlated ($r = 0.968$, $n = 285$) with the integrated radiant energy values obtained by an Eppley pyrheliometer. A more nearly linear correspondence between the two types of instruments is obtained if the response of the pyrheliometer is corrected for the effect of the declination of the sun.

The authors wish to express their appreciation to Dr. W. C. Kelly, who assisted in the construction of these recorders, and to Dr. C. S. Brandt, who deduced the relationship used to correct the pyrheliometer readings for the effect of the declination of the sun.

U. S. PLANT, SOIL, AND NUTRITION LABORATORY
AGRICULTURAL RESEARCH ADMINISTRATION
U. S. DEPARTMENT OF AGRICULTURE
ITHACA, NEW YORK

LITERATURE CITED

1. HAMNER, K. C. and MAYNARD, L. A. Factors influencing the nutritive value of the tomato. A review of the literature. U. S. Dept. of Agriculture Misc. Publ. **502**. 23 pp. 1942.
2. MAYNARD, L. A. and BEESON, K. C. Some causes of variation in the vitamin content of plants grown for food. Nutrition Abst. and Rev. **13**: 155–164. 1943.
3. SOMERS, G. F. and BEESON, K. C. The influence of climate and fertilizer practices upon the vitamin and mineral content of vegetables. A review of the literature. Advances in Food Research **1**: 291–324. 1948.
4. SOMERS, G. F., HAMNER, K. C., and KELLY, W. C. Further studies on the relationship between illumination and the ascorbic acid content of tomato fruits. Jour. Nutr. **40**: 133–143. 1950.
5. SPRAGUE, V. G. and WILLIAMS, E. M. An inexpensive light recorder. Plant Physiol. **16**: 629–635. 1941.
6. SPRAGUE, V. G. and WILLIAMS, E. M. A simplified integrating light recorder for field use. Plant Physiol. **18**: 131–133. 1943.
7. WALLACE, R. H. Methods of sampling visible radiation. Plant Physiol. **12**: 647–666. 1937.

BOUND WATER IN *ASPERGILLUS NIGER*

GLENN W. TODD AND J. LEVITT

(WITH TWO FIGURES)

Received August 28, 1950

The concept of bound water has been defined and explained in many ways (**2**). It is not the purpose of the present investigation to extend the controversy, but simply to find out whether physiological changes produced in plants as a result of exposure to specific environmental vapor pressures can be explained in terms of a binding of water by (or hydration of) specific substances in the plant.

Evidence has repeatedly been produced both for and against a relationship between bound water and both frost and drought resistance (**2, 4, 5**). Two main difficulties have prevented the resolution of this point: (a) the use of inadequate methods that may be measuring something other than bound water (**3**) and (b) the use of higher plants whose cells have large vacuoles capable of storing high concentrations of sugars and other non-protoplasmic substances that may conceivably bind water. Any binding of water by protoplasmic substances under these circumstances would be difficult to detect.

In the present investigation these difficulties have been overcome (a) by using a very simple but accurate method of measuring bound water, (b) by using a microorganism whose cells contain very little vacuolar material and therefore consist very largely of protoplasmic substances. The organism is also capable of growth in a wide range of vapor pressures.

Methods

A. CULTURAL.—Flasks containing the necessary quantity of Steinberg's dibasal medium (50 ml. in 125-ml. flasks or 200 ml. in one-liter flasks) were autoclaved at 15 pounds pressure for 20 minutes. The cooled media were inoculated with spores from a pure culture of *Aspergillus niger*. The mycelium was allowed to grow for about seven days at 25° C, before harvesting. The osmotic pressure of the solutions was controlled by adding the necessary amounts of dextrose (from 50 to 547 g./l.).

B. OSMOTIC PRESSURE DETERMINATIONS.—Two methods were used to determine the osmotic pressures of the culture media: (1) by measuring the freezing-point lowerings of the solution, using either a Beckmann thermometer or a copper-constantin thermocouple connected to a Leeds and Northrup Type K potentiometer and a galvanometer; (2) by calculation from the concentrations of sugars, using the values given in the International Critical Tables.

C. BOUND WATER DETERMINATIONS.—At the end of the growing period, the mycelial mats were harvested, washed, blotted, then dried in weighing

bottles over a desiccant (Al_2O_3 or $Mg(ClO_4)_2$) in an evacuated desiccator at room temperature until equilibrium was attained (one to two months). The weighing bottles were then transferred to an oven at 80° C or 110° C and again dried until equilibrium was reached (one to two months). The difference between these two weights was taken as the bound water, which was expressed as a per cent. of the oven-dry weight. Bound water determined by this method is simply the water held by the mycelium at room temperature in a vacuum but driven off at 80° C or 110° C respectively.

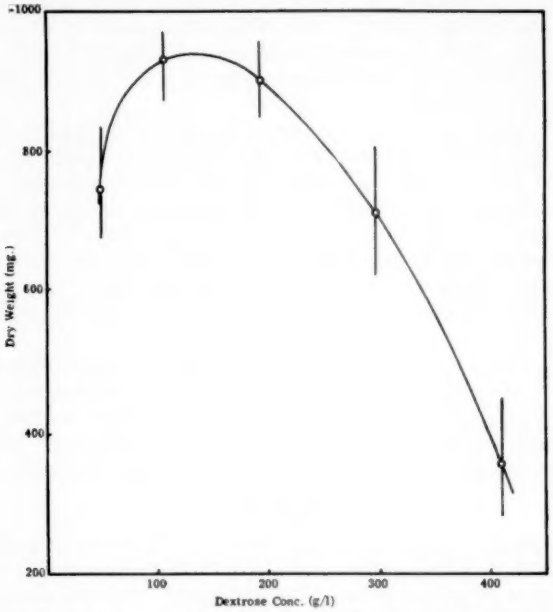


FIG. 1. Growth of *A. niger* in culture media containing various amounts of dextrose. Each point is an average of three determinations, the spread being shown by the straight line through points.

Experimental methods

A. OSMOTIC PRESSURES OF CULTURE SOLUTIONS.—There was good agreement between the values obtained from freezing-point determinations and those calculated from the sugar concentration. The calculated values were always about 10% higher, and are adopted in the following experiments, since they are not open to complicating factors such as undercooling and possible crystallizing out of solute which may occur during freezing-point determinations.

B. GROWTH OF THE ORGANISM.—Figure 1 shows the actual amounts of growth obtained at the different osmotic pressures using dextrose. Microscopic observations showed that the cells were much smaller in the solutions of higher osmotic pressures. A few small vacuoles were observed in each cell when grown at the lowest osmotic pressure, but none could be detected in the cells grown at the higher osmotic pressures.

C. BOUND WATER OF FUNGUS MYCELIUM GROWN AT DIFFERENT OSMOTIC PRESSURES.—Table I shows the results obtained with cultures grown in triplicate at six different osmotic pressures and dried over Al_2O_3. Although

TABLE I

BOUND WATER OF *A. niger* MYCELIUM IN RELATION TO OSMOTIC PRESSURE

O.P. of medium (in atm.)	Sporulation*	pH of medium at harvest**	Dry weight at 110° C (in mg.)	Bound water content at room temperature (in mg.)	Bound water %
7.7	1	2.6	396.4	31.4	7.9
7.7	3	2.5	290.8	24.3	8.4
7.7	3	2.7	286.2	20.2	7.1
15.5	3	2.6	420.8	28.2	6.7
15.5	3	2.5	397.9	25.4	6.4
15.5	5	2.8	413.4	30.4	7.4
30.6	5	2.4	470.5	38.0	8.1
30.6	4	2.4	409.8	35.3	8.6
30.6	4	2.6	327.8	20.9	6.4
52.7	2	2.3	442.0	78.8	17.8
52.7	1	2.4	250.7	42.3	16.8
52.7	3	2.5	370.6	28.6	7.6
83.7	0	2.2	371.1	120.4	32.4
83.7	0	2.5	64.6	15.8	24.5
83.7	0	2.4	184.1	41.5	22.5
132.2	0	2.1	273.3	73.1	26.7
132.2	0	2.1	260.4	74.2	28.5
132.2	0	2.1	319.1	97.1	30.4

*Sporulation: 5 = heavy; 0 = none.
**Original pH 4.4–5.6.

the three series of cultures at the lowest osmotic pressures contained about the same amount of bound water, the four grown at the highest osmotic pressures showed a direct relation between osmotic pressure and bound water.

In the next experiment $Mg(ClO_4)_2$ was used instead of Al_2O_3 and since the former maintains a lower vapor pressure (1), one would expect lower bound water values, and this is exactly what was obtained (fig. 2). Furthermore the bound water is greater when the mycelium is dried at 110° C than when it is dried at 80° C. But in these two cases the correlation is even better. Only the culture at the highest osmotic pressure shows a bound

water value (average 16.58) that is out of place. This series of cultures, however, had to be grown twice as long as the others in order to obtain enough mycelium and is therefore not strictly comparable.

There is thus no doubt that the water bound by the mycelium of *Aspergillus niger* increases with an increase in the osmotic pressure of the medium in which it is grown.

In the case of higher plants (**5**) such correlations can be easily explained

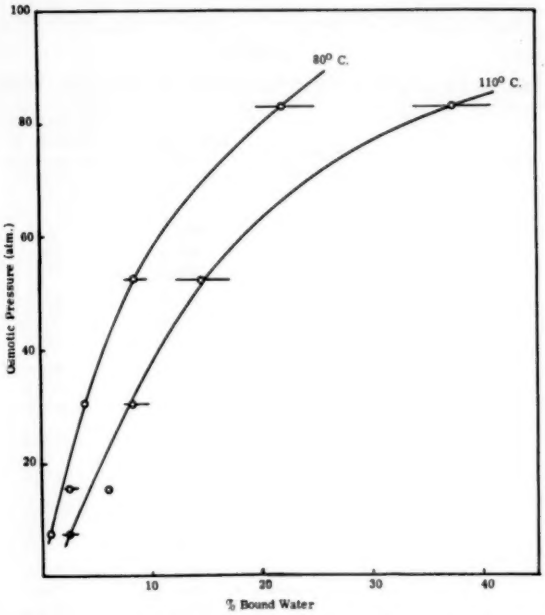


FIG. 2. The relationship between per cent. bound water of *A. niger* mycelium and osmotic pressure. Each point is an average of three determinations, the spread being shown by the straight line through points.

$$\% \text{ Bound Water} = \frac{\text{d.w. at room temp.} - \text{d.w. at 80° C (or 110° C)}}{\text{d.w. at 80° C (or 110° C)}} \times 100$$

by the increased sugars (**4**). It is therefore important to investigate this possibility in *A. niger*. Although the mycelium was well washed before drying, it is conceivable that small amounts of sugar remained in the case of the mats grown at higher concentrations. Furthermore, in spite of the paucity of vacuoles, the cells may conceivably accumulate some sugar. It should be pointed out, however, that in order for sugars to account for the high bound water of the mycelium grown at high osmotic pressures, the

sugar itself would have to bind even larger amounts, since the mycelial values would be the averages between low-binding non-sugar and high-binding sugar molecules.

In order to investigate this possibility, aliquots of culture medium were treated in the same way as the mycelium. Table II shows the results. The

TABLE II

BOUND WATER OF THE CULTURE MEDIUM USED FOR OSMOTIC PRESSURE STUDIES

O.P. of medium (in atm.)	Sugar concentration of medium (g/l.)	Dry weight at 80°C (in mg.)	Bound water content at room temperature (in mg.)	% Bound water
7.7	50	87.8	10.6	12.1
15.5	106	199.5	17.7	8.9
30.6	193	365.9	10.4	2.8
52.7	297	548.2	14.9	2.7
83.7	410	762.3	16.8	2.2
132.2	547	917.9	6.8	0.7

highest value was much less than the highest value obtained for the mycelium. Furthermore, the relation to osmotic pressure was the reverse of that obtained with the mycelium, *i.e.*, the medium of lowest concentration had the highest bound water. This may possibly be due to the higher proportion of mineral salts or perhaps partly to the greater tendency for the more concentrated sugar solutions to form an impermeable surface crust. In any case, the relation between the water bound by the mycelium and the osmotic pressure of the medium in which it is grown cannot possibly be explained by the sugars.

The only substances that are both capable of binding large amounts of water and are present in the mycelium in sufficient quantity to account for the increase in bound water are the proteins. Further investigations are being made to determine whether or not this point can be proven.

Summary

1. The term bound water is used for water held at room temperature in an evacuated desiccator containing a desiccant, but driven off in an oven at 80° C or 110° C respectively.

2. The quantities of such bound water held by the mycelium of *Aspergillus niger* varied directly with the osmotic pressure of the solution in which it was grown.

3. These differences could not be accounted for by an accumulation of sugars since the sugars bind far less water than the mycelium grown at higher osmotic pressures.

UNIVERSITY OF MISSOURI
COLUMBIA, MISSOURI

LITERATURE CITED

1. Bower, J. Comparative efficiencies of various dehydrating agents used for drying gases. Bur. Standards, Jour. Res. **12**: 241–248. 1934.
2. Gortner, R. A. Outlines of Biochemistry. John Wiley and Sons, New York. 1938.
3. Grollman, A. J. The vapor pressure of aqueous solutions with special reference to the problem of the state of water in biological fluids. Jour. Gen. Physiol. **14**: 661–683. 1931.
4. Levitt, J. Frost Killing and Hardiness of Plants. Burgess Publ. Co., Minneapolis, Minn. 1941.
5. Newton, R. and Martin, W. M. Physico-chemical studies on the nature of drought resistance in crop plants. Canadian Jour. Res. **3**: 336–427. 1930.

THE STEROLS OF ALFALFA AND CERTAIN CEREAL GRASSES

E. H. Blair,[1] H. L. Mitchell and Ralph E. Silker

(WITH SIX FIGURES)

Received September 18, 1950

Little attention has been given to the sterols of the vegetative portion of green plants. This perhaps has been due partially to the difficulty of determining sterols quantitatively in the presence of chlorophyll and carotenoids. With an increase in the use of certain sterols for the production of pharmaceuticals, attention has been directed again to the sterols of green plant tissue, with methods being developed to facilitate their estimation (**5**). The study reported herein was undertaken to determine the nature and the amount of the sterols in the lipid fraction of some crops which are suitable for commercial dehydration, for such dehydrated material would be the logical source for the production of sterols from green plants if they are found to be economically important.

Determination and characterization of sterols

Fresh plant tissue was autoclaved for five minutes at 10 pounds pressure, and dried at 65° C for four hours. The dried material was ground to pass through a 20-mesh screen. Sterols were determined quantitatively by the colorimetric method of Wall and Kelley (**5**), which consists of extracting the dried plant tissue with Skellysolve B, removing chlorophyll and xanthophyll by adsorption, precipitating carotene with iodine, precipitating the sterols with digitonin, and subjecting the resulting sterol digitonide to the Liebermann-Burchard reaction. A standard curve was established for each plant tissue to be studied by isolating the sterol with digitonin (**5**) and dissolving a weighed amount of the sterol digitonide in acetic acid. Aliquots of this solution were subjected to the Liebermann-Burchard reaction, and the intensity of the color produced was measured with a Beckman spectrophotometer. The digitonide of alfalfa sterol has an absorption maximum at 680 mμ (**5**). Absorption maxima for the digitonides of the sterols of brome grass and the wheat plant were found at 680 mμ also (fig. 1). All readings accordingly were made at 680 mμ 30 minutes after the reaction was started.

Sterols may exist in the lipid fraction of plant tissue in a combined condition as esters, and when combined are not determined by the method used above (**4**). To determine total sterols, it is necessary to saponify the Skellysolve B extract of the plant tissue and use the unsaponifiable fraction in the Wall and Kelley procedure. Alfalfa does not contain combined sterols (**5**). Nothing was found in the literature, however, concerning the condition of

[1] Contribution No. 406, Department of Chemistry, Kansas State College. This work was supported by the Kansas Industrial Development Commission.

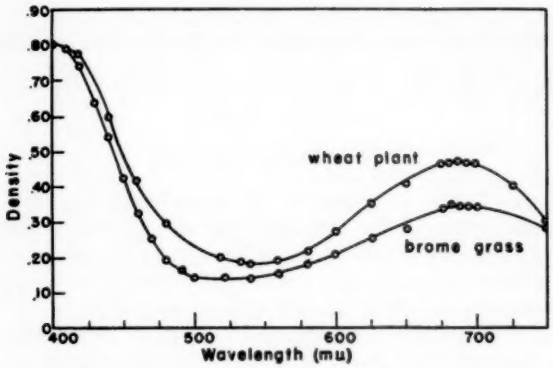


FIG. 1. Absorption curves of the digitonides of the sterols in brome grass and wheat plant after Liebermann-Burchard reaction.

the sterols in brome grass and the wheat plant. Accordingly, both free and total sterols were determined on each sample.

When subjected to the Liebermann-Burchard reaction, alfalfa sterol digitonide was reported by Wall and Kelley to have a time-density curve characteristic of spinasterol; *i.e.*, a maximum color density was produced immediately upon addition of the reagents. The density decreased with time, and approached constancy at 30 minutes. The $E^{1\%}_{1\ cm.}$ value for the sterol digitonide obtained in this work was 77.3 as compared with a value of 79.2 obtained by Wall and Kelley. Wall and Kelley state that the predominant sterol of alfalfa is probably spinasterol. FERNHOLZ and MOORE (**2**) isolated a sterol from alfalfa and characterized it as α-spinasterol. A sterol was isolated from alfalfa in this laboratory by the method of Fernholz and Moore. The physical constants and derivatives agreed with those of α-spinasterol. The digitonide of this sterol had an $E^{1\%}_{1\ cm.}$ value of 108.5 at 680 mμ 30 minutes after the addition of the Liebermann-Burchard reagent. This $E^{1\%}_{1\ cm.}$ value agrees with the Wall and Kelley value of 110 for spinach spinasterol digitonide. No data were obtained which would account for the difference in the $E^{1\%}_{1\ cm.}$ value of 108.5 for the digitonide of the sterol isolated from alfalfa by the Fernholz and Moore method and the value of 77.3 obtained with the material which was isolated with digitonin for preparation of the standard curve. This difference may be due to the presence of sterols other than spinasterol which are precipitated by digitonin, but which are lost in the Fernholz and Moore isolation method. It may be due also to the precipitation by digitonin of other hydroxy compounds as well as sterols (**3**), which would result in a low $E^{1\%}_{1\ cm.}$ value.

The time-density curve for brome-grass sterol digitonide when read at 680 mμ is markedly different from that of alfalfa sterol digitonide (fig. 2). The color density is at a minimum in the early stage of the reaction and is

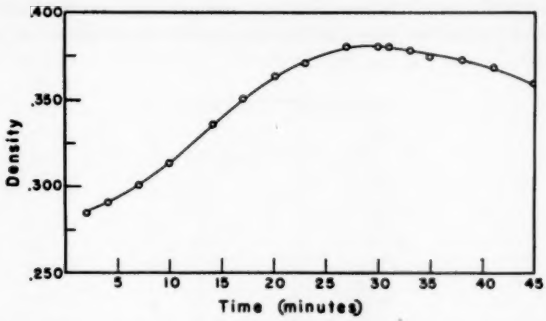


FIG. 2. Time-density curve for the sterol digitonide of brome grass after Liebermann-Burchard reaction.

at a maximum at about 30 minutes. This type of reaction is characteristic of the sitosterols (**5**). Because of these differences in rate of color development, it was necessary to prepare a standard curve for each plant which was analyzed.

The $E^{1\%}_{1\ cm.}$ value for brome grass sterol digitonide was found to be much lower than the reported value for β-sitosterol. The Liebermann-Burchard reaction is specific for unsaturated sterols, whereas digitonin precipitates both saturated and unsaturated sterols (**1**). The possibility that this lower $E^{1\%}_{1\ cm.}$ value might be due to the presence of saturated sterols was investigated, using the method of ANDERSON and NABENHAUER (**1**) for estimating saturated sterols. A small amount of saturated sterol was found, and with this correction, an $E^{1\%}_{1\ cm.}$ value of 30.0 was obtained. The $E^{1\%}_{1\ cm.}$ value of β-sitosterol is 29.2 (**5**).

Sterols were isolated from 5 kg. of dehydrated brome grass by the method of FERNHOLZ and MOORE (**2**). The final product was recrystallized three times from methyl alcohol. A yield of 500 mg. was obtained. It had a melting point of 136–137° C which did not change on further recrystallization. The sterol acetate melted at 127–128° C, and the benzoate at 144–146° C. On the basis of these melting points and the time-density curve of the digitonide the sterol which was isolated from brome grass was considered to be β-sitosterol.

An investigation also was made of the nature of the wheat plant sterols. The time-density curve obtained with the digitonide of this sterol was similar to the curve for brome grass sterol digitonide. It, too, appeared to be a sitosterol. The $E^{1\%}_{1\ cm.}$ value was 26.6. When examined for saturated sterols, only a trace was found.

Effect of stage of growth and fertilization on sterol content

Figure 3 shows the effect of stage of growth on the sterol content of alfalfa. The last sample was taken when the plants were in early bloom.

Only free sterols were found, which confirms the data of Wall and Kelley. It will be seen that there was a small decrease in sterol content as the plants became older.

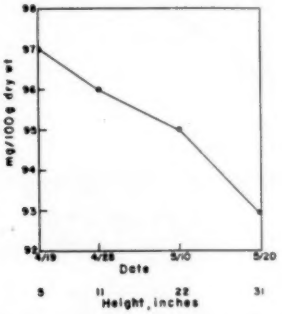


FIG. 3. Effect of stage of growth on the sterol content of alfalfa.

Figure 4 shows the effect of stage of growth on the sterol content of brome grass which had been fertilized with 100 pounds of nitrogen per acre, the nitrogen being applied as ammonium nitrate. A considerable proportion of the sterol was found to be combined, the proportion increasing as the plants approached maturity. Total sterols increased slightly during growth of the plants.

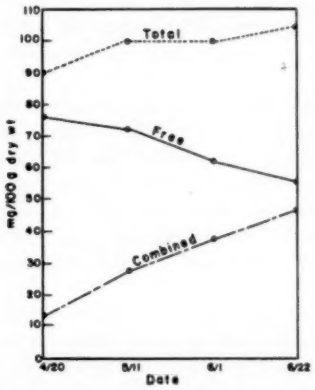


FIG. 4. Effect of stage of growth on the sterol content of brome grass.

Samples were obtained from brome grass plots to which ammonium nitrate had been applied at varying rates. Samples were collected at the time the plots were cut for hay after vegetative growth had ceased. In fig-

ure 5 it will be seen that both free and total sterols increased as the nitrogen application was increased from zero to 200 pounds per acre. Slightly more than half of the sterols were in combined form in each case.

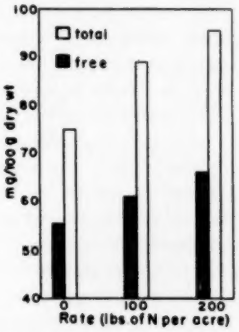


FIG. 5. Effect of fertilization with ammonium nitrate on the sterol content of brome grass.

In figure 6 is presented the effect of stage of growth on the sterol content of the wheat plant (Pawnee variety) sampled at 3-week intervals. The final sample was taken shortly after heads began to appear. Combined sterols were found in the last sample only, where they comprised about 25%

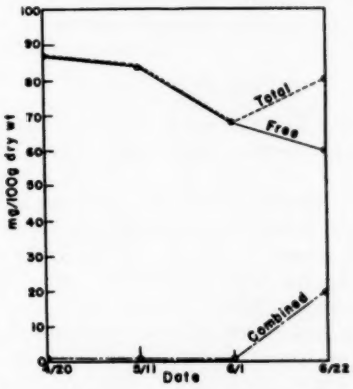


FIG. 6. Effect of stage of growth on the sterol content of the wheat plant.

of the total sterols. Free sterols decreased throughout the growth period. Since heads were present when the last sample was obtained, perhaps this part of the plant contributed the combined sterols that were found. This

might account also for the reversal of the downward trend of the total sterols that occurred at the time heads were formed.

Summary

Factors which influence the sterol content of alfalfa, brome grass, and the wheat plant were studied. Free sterols decreased as the plants approached maturity, while combined sterols of brome grass increased. Combined sterols were not detected in alfalfa at any time during its growth up to the early bloom stage. The wheat plant contained no combined sterols until heads began to appear, at which time 25% of the sterols were in combined form. Fertilization of brome grass with ammonium nitrate caused an increase in both free and combined sterols.

From the shape of the time-density curves of the sterol digitonides when subjected to the Liebermann-Burchard reaction, the sterols of brome grass and the wheat plant appeared to be sitosterols. A sterol having a melting point of 136–137° C was isolated from brome grass. The melting points of its acetate and benzoate indicated it to be β-sitosterol. Suggestions were made to explain the difference in $E^{1\%}_{1\ cm.}$ values of the digitonide of alfalfa sterol isolated by the Fernholz and Moore method and that isolated by precipitation with digitonin.

The authors are indebted to the W. J. Small Co., Inc., Kansas City, Mo., for supplying the dehydrated alfalfa meal for sterol isolation. The Department of Agronomy at Kansas State College furnished the fresh plant materials which were used in this investigation.

KANSAS AGRICULTURAL EXPERIMENT STATION
MANHATTAN, KANSAS

LITERATURE CITED

1. ANDERSON, R. J. and NABENHAUER, F. P. The separation of unsaturated from saturated sterols. Jour. Amer. Chem. Soc. **46**: 1957–1960. 1926.
2. FERNHOLZ, E. and MOORE, M. L. The isolation of α-spinasterol from alfalfa. Jour. Amer. Chem. Soc. **61**: 2467–2468. 1939.
3. FIESER, L. F. and FIESER, M. Natural products related to phenanthrene. Third Edition, A.C.S. Monograph 70, p. 579. Reinhold Publishing Corp., New York. 1949.
4. SCHOENHEIMER, R. and SPERRY, W. M. A micromethod for the determination of free and combined cholesterol. Jour. Biol. Chem. **106**: 745–760. 1934.
5. WALL, M. E. and KELLEY, E. G. Determination and nature of leaf sterols. Anal. Chem. **19**: 677–683. 1947.

SOME EFFECTS OF DECAPITATION ON ELECTRICAL AND ELONGATION PHENOMENA IN THE AVENA COLEOPTILE[1]

KENNETH S. MILLS AND A. R. SCHRANK

(WITH FOUR FIGURES)

Received October 5, 1950

Introduction

Growth of the Avena coleoptile, including elongation and curvature responses, is dependent on the auxin which is produced only by the cells in the extreme apex (**4, 12, 13**). A polar force is required to direct and control the distribution of this auxin. It frequently has been suggested that the inherent electrical field could function as this force and there is evidence that is consistent with this thesis (**9**). However, if the inherent electrical field is dependent on the auxin-regulated elongation process, then this hypothesis would be untenable. For this reason, it is important to test this possibility by experimentation.

Preliminary evidence, from experiments which were not specifically designed to evaluate this point, indicates that the longitudinal electrical polarity of the Avena coleoptile is not directly dependent on the presence of auxin that is required for elongation (**4, 14**). It also has been demonstrated that a transverse electrical polarity, in response to mechanical and electrical stimulation, can be established in the etiolated oat seedling in which the auxin has been decreased by removal of the apex before stimulation (**7, 10**). However, no previous attempt has been made to measure simultaneously the elongation rate, as a manifestation of the functional auxin content, and the longitudinal electrical polarity of the Avena coleoptile.

It is generally accepted that decapitation of the coleoptile removes the source of auxin and results in its gradual decrease in the remaining tissue, because auxin disappears in the process of growth (**2**). Since growth of the coleoptile is proportional to the amount of auxin added (**2, 11, 13**), it is permissible to use the rate of elongation as an index of the quantity of auxin used in this process. The present experiments were performed to determine the effects of decreased auxin supply resulting from decapitation, as measured by the elongation rate, on the longitudinal component of the electrical pattern of the coleoptile.

Materials and methods

The experimental material was the coleoptile of the Victory strain of *Avena sativa* (U. S. Department of Agriculture, C.I. 2020).[2] All seeds were

[1] Aided by funds from the University of Texas Research Institute.

[2] Supplied by the U. S. Department of Agriculture.

husked, soaked in tap water with the pH adjusted to 7.1 by a phosphate buffer and sprouted in Petri dishes on filter paper moistened with buffered tap water. The Petri dishes were kept 38 cm. from a 60-watt lamp (dark Ruby Mazda which emitted only wave lengths longer than 5,700 Å). They were then placed in a dark box in an aquarium containing the same buffer solution. Only plants that had a good general appearance, straight coleoptiles and fully developed root systems were selected for experimentation. In the apparatus, each seedling was placed in a holder similar to that in the aquarium and supported in the same buffer solution. After handling, before an experiment, the plant was always allowed to rest at least 30 minutes to recover from the mechanical stimulation.

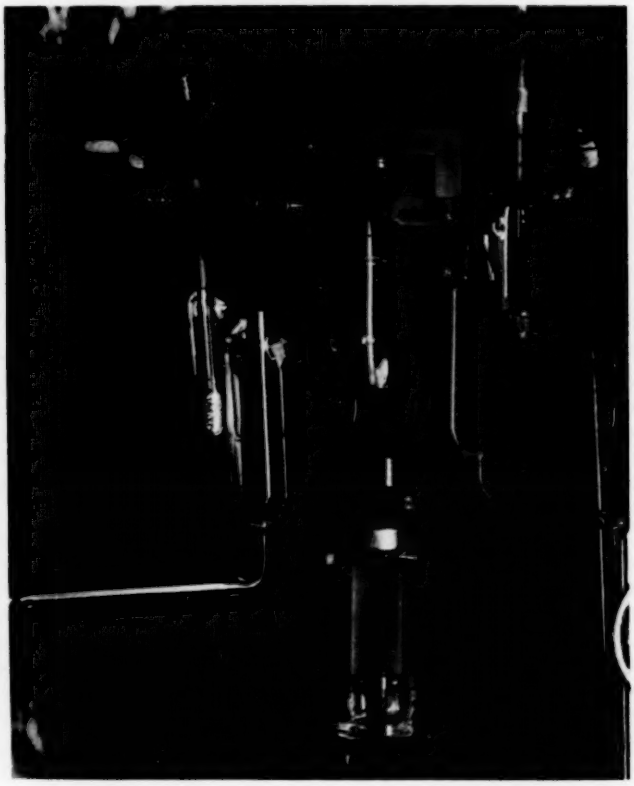


FIG. 1. Photograph of experimental equipment (see text for description).

The electrical measuring device was a duBridge (5) amplifier in a light-

tight box with a Rubicon galvanometer (Model 3403 HH) as the indicating instrument. In this circuit the galvanometer had a sensitivity of 1.08 millivolts per scale division. Variation of the zinc–zinc sulphate electrodes was never more than 2.2 millivolts during an experiment. The electrodes were placed in glass cups which were filled with Shive's solution made with distilled water. Figure 1 shows the spatial relationship of the coleoptile to the contacts and attached glass cups. In all instances the contacts were placed so as to obtain the maximum longitudinal electrical polarity but to maintain approximately uniform lengths of included segments. The apical contact was always connected to the control grid and the basal contact was grounded. Glass ring contacts and the combination contact-holder were used for the isolated coleoptiles (sheaths separated from primary leaves and seed). Actual contact with the tissue was made by a water meniscus.

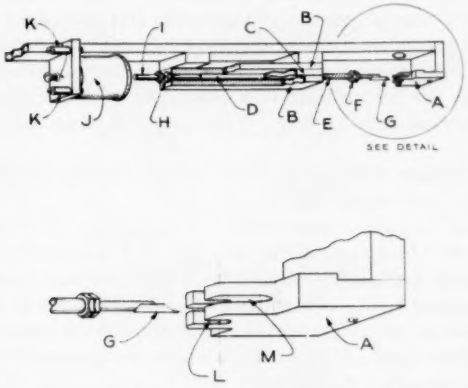


FIG. 2. Mechanical decapitator (see text for description).

Elongation was measured by observing a spot of India ink, placed near the apex, with a horizontal microscope equipped with a Filar micrometer ocular.

All experiments were performed in a dark room equipped with a temperature- and humidity-controlling device, which maintained the temperature within $\pm$ 3 of 24° C. The temperature never varied more than 1° C during an experiment. A ruby glass neon tube, which emitted only wave lengths longer than 6,200 Å, was the light source.

Various attempts at decapitation in preliminary experiments made it evident that some device was needed to permit precise control of decapitation of the coleoptile with little effect on the electrical polarity. (Curve II in figure 3 B shows the type of electrical response that is inevitable when the plants are decapitated by hand.) Figure 2 shows the construction details of a mechanical decapitator designed to meet the essential requirements.

On one end of the brass bar was a small block of lucite (A), which had two grooves. The vertical groove (L) was slightly larger than the coleoptile and was designed to encircle it on three sides during decapitation to prevent movement to either side or away from the blade. Groove (M) was horizontal to allow the passage of the decapitator blade through the plant.

A second unit suspended from the brass bar consisted of two $\frac{3}{8}$ by $\frac{1}{8}$ inch brass blocks (B), three inches in length, and three $\frac{1}{8}$ inch square iron rods. Two of the iron rods (C) were screwed between the brass blocks (B) so that a square hole remained down the center of this combination. The square rod (D), six inches long, was lapped to fit snugly into this hole, thus eliminating sway in the movable decapitator rod. This sliding rod (D) was rounded and threaded for one-half inch on each end. A compression spring (E) was placed on the blade end of the shaft. Nuts (F), placed in front of the spring, permitted adjustment of tension. The knife (G), part of a thin razor blade, was mounted at an angle with DeKhotinsky cement. Two nuts (H) were placed on the back thread of this movable bar (D) to allow adjustment of the cutting depth. A soft iron cap (I) was drilled and tapped to fit the end of the threaded bar (D) to serve as a bumper and to add surface for the electromagnet to hold. Each adjustment was held securely by a locking nut.

The third element of this decapitator was an electromagnet (J) with its wires connected to terminals (K).

In operation, the decapitator circuit was completed, the cutter mechanically cocked, the blade and plastic coleoptile rest moistened with distilled water, the cutting depth adjusted, and the decapitator moved onto the coleoptile at the desired level. Movement of the decapitator was accomplished by a micromanipulator. Opening the circuit allowed the spring to drive the blade through the plant. The circuit was then recompleted, the cutter recocked and the decapitator removed.

Experimental

Effect of decapitation on rate of elongation and longitudinal electrical polarity

In this series intact coleoptiles 34 ($\pm$ 2) mm. in length were used. The ink spot was placed about 5 mm. below the apex. Figure 3 A shows the characteristic elongation rates (measurements at 5-minute intervals). Curve I is a typical example of six experiments in which the plants were not decapitated. The gradual decrease in the rate of elongation is normal for coleoptiles of this age and length.

Curve II of figure 3 A was chosen as the typical representative of eight experiments. In this set the coleoptiles were decapitated by hand, using a quick stroke with a thin razor blade and supporting the opposite side of the apex with a finger. Three ($\pm$ 2) millimeters of the apex were removed. The initial rate of elongation, prior to decapitation, is less than that shown by the control curve I. This is due to inherent variation of the individual

coleoptiles. After decapitation (indicated by an arrow in the figure) and lasting for 80 minutes, there is a period of slight decrease in the rate of elongation. From 80 to 155 minutes after decapitation the rate is greatly decreased. This is followed by increased elongation beginning 155 minutes after decapitation.

Curve III of figure 3 A is the characteristic example of five experiments. Coleoptiles were mechanically decapitated as described in the previous section (decapitated segment length, 3 to 4 mm.). The initial rate of elongation is practically equal to that of the control curve I. After decapitation the sequence of rate changes is the same as in curve II, but the time inter-

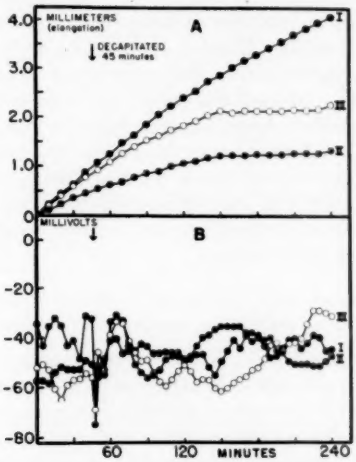


FIG. 3. Effects of decapitation on rates of elongation and on magnitude and orientation of the longitudinal electrical polarity. 3A. Elongation changes. 3B. Electrical changes in the same plants. Curves I, Apical cells not removed. Curves II, Apical cells removed by hand. Curves III, Apical cells removed by mechanical decapitation.

vals are slightly different. These changes in rates of elongation (curves II and III) are in general agreement with earlier work (**4, 12**).

Figure 3 B shows the concurrent electrical polarities of the same plants whose elongation rates were presented in figure 3 A. The differences in magnitudes of electrical polarities shown in figure 3 B are due to individual variation in the plants. Two other phenomena manifested by these curves warrant consideration.

First, in curve II there is a large rapid increase in the electrical polarity immediately after decapitation, but the voltage always recedes to the original level within a few minutes. In other experiments of this series the polarity frequently decreases beyond the original average before returning to this level. These electrical changes are apparently the result of mechani-

cal stimulation caused by bumping the coleoptile against the apical contact in the process of manual decapitation. In curve III (coleoptile mechanically decapitated) the initial electrical response is greatly reduced, while in other experiments of this kind it was completely eliminated.

A second feature, appearing in the curves in figure 3 B, is the gradual decrease of the electrical polarity shown by curves I and III toward the end of the experiment. This is also apparent in some of the duplicate curves, which are not included. However, in every case the decline in polarity started only after the decrease in the elongation rate was noted.

Several observations can be made from the data in figure 3. A large difference in the initial rates of elongation is shown by curves II and III, while the initial electrical polarity indicated by curve II is only slightly less than is shown by curve III. There is always a decrease in the rate of elongation of the decapitated plants but never a concurrent decrease in the longitudinal polarity. Toward the end of the experiment the decapitated plants show an increased elongation rate due to a functional regeneration of the apical cells (**3**). A parallel increase in the electrical polarity was never observed. These results clearly indicate that the longitudinal electrical polarity is independent of the auxin-controlled elongation rate. Results of a corresponding series of experiments on isolated coleoptile sheaths are in agreement with these observations.

Effect of mechanical stimulation of cutting on rate of elongation and longitudinal electrical polarity

Previous investigations have demonstrated that the electrical pattern of Avena is sensitive to mechanical stimulation (**7**). The data in figure 3 show that the initial electrical response caused by manual decapitation can be substantially reduced by use of a mechanical decapitator. One other remote but real possibility remains. Theoretically, a prolonged response to the mechanical stimulation of cutting could be equal but opposite in orientation to the effect of the absence of the apical cells with the net result that the average magnitude of the electrical polarity would remain unchanged. The present experiments treat this possibility.

Intact plants 33 ($\pm$ 1) mm. long were used. The decapitator was adjusted so the blade would cut only $\frac{3}{4}$ of the way through the coleoptile. The ink spot was placed about 5 mm. from the apex while the level of the cut was 3 ($\pm$ 1) mm. below the apex. Preliminary tests had shown that the best way to keep the cut wet and to keep its edges from spreading apart was to place a piece of wet filter paper (4 mm. long and 2 mm. wide) over the cut so that the paper strip would extend above the apex. Distilled water was added to the filter paper strip from time to time and thus to the incision. This arrangement functioned as a diffusion bridge for the transport of auxin from the apex to the basal cells.

Curve Ia in figure 4 A is another one of the control series. It shows a uniform rate of elongation throughout the 240-minute period. Curve IV,

representative of six experiments, is from a "partially decapitated" intact plant in which the incision was kept wet as previously indicated. This curve shows only a slight decrease in the elongation rate during the period of 210 minutes. Some of the control curves (curve I, fig. 3 A) from coleoptiles that were not decapitated show decreases of similar magnitude in the rate of elongation.

The corresponding curves in figure 4 B represent the longitudinal electrical polarities of the same plants. Curve Ia, the control, maintains approximately the same average magnitude for the 240-minute period. Curve IV

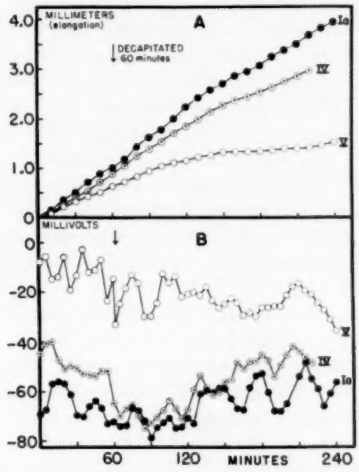


FIG. 4. Effects of decapitation or mechanical stimulation of cutting on rates of elongation, magnitude, orientation and variability of longitudinal electrical polarities. 4A. Elongation changes. 4B. Electrical changes in the same plants. Curves Ia, Apical cells not removed. Curves IV, Incision cut into apex with apical cells not removed. Curves V, Apical cells of young (short) plant removed by mechanical decapitator.

shows an increase in the potential difference after decapitation which lasts for a period of about 55 minutes. Then there is a gradual decrease in the E.M.F. toward the end of the experiment. However, there is no consistent difference between these electrical curves and those in figure 3 B.

The results of these experiments (uniformly supported by a parallel series using isolated coleoptiles), in comparison to the data in figure 3, demonstrate that mechanical stimulation of cutting does not have exactly the opposite effect of removing the tip in so far as the potential difference is concerned. Therefore, the maintenance of the longitudinal electrical polarity is not a prolonged result of mechanical stimulation, nor is it in any way dependent on the presence of the apical cells of the coleoptile.

Effect of decapitation on the spontaneous variation of the longitudinal electrical polarity

Continuous bioelectrical fields manifest spontaneous variations in magnitude even though all known experimental conditions are constant. Curve III in figure 3 B and curve IV in figure 4 B indicate that neither the absence of the apex nor the mechanical stimulation of decapitation influences the inherent variability of the electrical polarity. Since shorter (younger) plants exhibit greater electrical variation (**14**), they could conveniently be used to check this observation.

Intact plants, **20** ($\pm$ 1) mm. in length, were selected. The ink spot was placed about **3** mm. from the apex, the apical ring contact **5** ($\pm$ 0.5) mm. below the apex, and the basal contact was placed so that a segment of tissue **12** ($\pm$ 2) mm. remained between the contacts. A segment **2** ($\pm$ 1) mm. long was removed. Each experiment was continued until there was first a decrease and then an increase in the elongation rate.

Curve V in figure 4 A, from a decapitated coleoptile, is typical of a group of four experiments. It is noted that the changes in the elongation rate are in the same sequence as in the corresponding previous curves. The total cycle is somewhat shorter for the 20-mm. coleoptile primarily because the duration of the first decreased rate, soon after decapitation, is shorter than for the longer plants.

Curve V in figure 4 B represents the electrical changes of a short decapitated plant. This curve shows a gradual increase in the magnitude of the polarity and indicates a decrease in electrical variability during the latter half of the experiment. However, the decrease in variability starts several minutes after the decreased elongation rate is evident, and the electrical variation does not reappear at the end of the experiment when an increase in the elongation rate is manifested. These results (supported by a parallel series using isolated coleoptiles) further indicate that the inherent variation of the electrical polarity is not directly dependent on the rate of elongation.

Discussion

Experimental results, which have recently been reported, demonstrate the magnitude, orientation and variability of the various components of the inherent electrical field of the Avena coleoptile (**14**). As an incidental part of this investigation the coleoptile was isolated from the seed, decapitated and then removed from the primary leaves. These data (**14**) indicate that such treatment had no effect on the longitudinal electrical polarity. Since it is not known how long after decapitation the electrical measurements were made, these observations cannot be evaluated in terms of effective auxin content of the tissue. The results in the present paper confirm some of the electrical pattern characteristics previously reported (**14**) and present simultaneous and continuous records of the functional auxin content (indexed by elongation rate) and the characteristics of the longitudinal electrical polarity before and after decapitation.

When the coleoptile was decapitated by hand, it was sometimes impossible to keep the plant from touching the apical contact. This mechanically stimulated region immediately became more negative with respect to the basal and grounded contact region. Such increase in negativity of the stimulated region is in agreement with results from the onion root (**6**), from Chara (**1**), and from Avena (**7**). Because of the orientation of the inherent electrical polarity, this stimulation resulted in a rapid increase in the potential difference. When the mechanical decapitator was used this initial electrical response was reduced from 75 to 100%. In a few experiments the initial increase in voltage was followed by a more gradual decrease. Evidence, currently available, is inadequate to reveal the nature of this second prolonged electrical change. It could be a "conduction phenomenon" such as reported for Chara (**1**). It might also be due to stimulation of the basal contact region or extended recovery from stimulation of the apical region.

These data prove that the absence of the apical cells causes no characteristic change in the magnitude or orientation of the longitudinal potential of intact coleoptiles or isolated sheaths. The possibility that a prolonged secondary electrical response to the mechanical stimulation of cutting is equal but opposite in orientation to the effect of the absence of the cells of the apex has been ruled out by experiment (fig. 4). The present findings also show that spontaneous variations in electrical polarity are not directly dependent on the presence of the apical cells. Therefore, it now appears that the origin and characteristics of the continuously maintained bioelectrical field are independent of the metabolic mechanism that is specifically responsible for elongation of the Avena coleoptile.

Summary

1. In order to investigate the effect of auxin on the longitudinal electrical polarity of the Avena coleoptile simultaneous measurements of elongation rates and voltages were made before and after decapitation.

2. A description of the decapitating instrument, devised to diminish mechanical stimulation of the coleoptile, is presented.

3. Decapitation of intact plants results in two decreased, but different, elongation rates which are followed by an increased rate. Elongation rates are used as an index of the functional auxin. No significant effect of decapitation on the orientation or magnitude of the longitudinal electrical polarity was observed. Isolated sheaths gave similar results.

4. The data show that the longitudinal electrical polarity of the Avena coleoptile is independent of the auxin-regulated elongation process.

5. Spontaneous variations of the longitudinal electrical polarity are also not directly dependent on the elongation mechanism.

Department of Zoology
University of Texas
Austin, Texas

LITERATURE CITED

1. Brown, Sidney O. Relation between light and electrical polarity of Chara. Plant Physiol. **13**: 713–736. 1938.
2. Bonner, J. and Thimann, K. V. Studies on the growth hormone of plants. VII. The fate of growth substance in the plant and the nature of the growing process. Jour. Gen. Physiol. **18**: 649–658. 1935.
3. Dolk, H. E. Concerning the sensibility of the decapitated coleoptiles of *Avena sativa* for light and gravitation. Proc. Kon. Akad. Wetensch. Amsterdam **29**: 1113–1117. 1926.
4. Dolk, H. E. Geotropie en Groeistof. Rec. trav. bot. néerl. **33**: 509–585. 1936.
5. Du Bridge, Lee A. and Brown, Hart. An improved DC amplifying circuit. Rev. Sci. Inst. **4**: 532–536. 1933.
6. Marsh, G. The effect of mechanical stimulation on the inherent E.M.F. of polar tissue. Protoplasma **11**: 497–520. 1930.
7. Schrank, A. R. Effect of mechanical stimulation on the electrical and curvature responses in the Avena coleoptile. Plant Physiol. **20**: 344–358. 1945.
8. Schrank, A. R. Electrical and curvature responses of Avena coleoptile to transversely applied current. Pp. 217–231 in Lund, E. J. and collaborators. Bioelectric fields and growth. University of Texas Press, Austin. 1947.
9. Schrank, A. R. Plant tropisms. Ann. Rev. of Plant Physiol. **1**: 59–74. 1950.
10. Schrank, A. R. and Backus, G. E. The relationship of auxin to the electrically induced growth responses in the Avena coleoptile. In press.
11. Thimann, K. V. and Bonner, J. The mechanism of action of the growth substance of plants. Proc. Roy. Soc. B. **113**: 126–149. 1933.
12. Went, F. W. Growth auxin and tropisms in decapitated Avena coleoptiles. Plant Physiol. **17**: 236–294. 1942.
13. Went, F. W. and Thimann, Kenneth V. Phytohormones. The Macmillan Co., New York. 1937.
14. Wilks, S. S. and Lund, E. J. The electrical correlation field and its variation in the coleoptile of *Avena sativa*. Pp. 24–74 in Lund, E. J. and collaborators. Bioelectric fields and growth. University of Texas Press, Austin. 1947.

AVAILABILITY OF SOIL MOISTURE FOR ACTIVE ABSORPTION IN DRYING SOIL[1]

THOMAS J. ARMY AND THEODORE T. KOZLOWSKI

(WITH FIVE FIGURES)

Received October 20, 1950

There is considerable disagreement as to whether or not water is equally available to plants in drying soil over the entire range from field capacity to wilting percentage. Some workers have repeatedly stated that water is equally available over this range (**5, 18, 23, 24**) while considerable data have accumulated which indicate that physiological processes in plants are altered as a result of decreasing soil moisture content even before the onset of wilting (**1, 2, 4, 6, 7, 12, 16, 21, 25**). As indicated by KRAMER (**15**) some of this disagreement exists because of differences in soil moisture tension–soil moisture content relations for different textural grades of soil. In certain coarse-textured soils most of the water in the range from wilting percentage to field capacity is held with a tension of less than one atmosphere and probably most of it is readily available. In some fine-textured soils, however, less than half of the available water may be held with a force of less than one atmosphere, and water is withheld from plants with appreciably greater energy over the lower part of the available range than over the upper part. Near the wilting percentage a small decrease in moisture content effects an enormous increase in tension.

Data also are available which indicate that active absorption and root pressure specifically are depressed by low soil moisture contents above the wilting percentage. LITVINOV and GEBHARDT (**17**) have reported a qualitative correlation between drying of soil and cessation of exudation from stumps of detopped plants. KRAMER (**14**) noted that removal of tops of unwilted herbaceous plants was not always followed by expected exudation. However, exudation usually began soon after the soil was watered. Working with coleus, sunflower and tomato, Kramer found that about 45% of the soil moisture between the moisture equivalent and the wilting percentage was unavailable to detopped plants. He concluded that the active absorption mechanism does not absorb against a diffusion pressure deficit greater than one or two atmospheres. The proportion of moisture in the "available range" over which exudation does not take place was essentially similar for clay, sand, and sandy loam. The uniformity of the results obtained indicated that the soil moisture content which limits exudation is determined predominantly by soil characteristics and not by plant characteristics. Using sunflower plants and a uniform, fertile sandy loam, MCDERMOTT (**19**) found that within a range of moisture content just above the wilting per-

[1] Contribution No. 781, Massachusetts Agricultural Experiment Station, Amherst, Massachusetts.

centage the detopped root systems exhibited negative exudation. As soil moisture content increased, the rate of negative exudation decreased, reached zero, and was followed by positive exudation which reached a maximum approximately at the moisture equivalent. At soil moisture contents higher than the moisture equivalent there was a decrease in exudation, and this was attributed to poor aeration and accompanying high carbon dioxide content. McDermott (**19**) found that an equation of the parabolic type expressing the relationship between the rate of exudation and soil moisture content best fitted the results obtained. He reported that about 60% of the soil moisture available to the whole plant when the soil was at the moisture equivalent was found to be unavailable to detopped root systems.

The present study was undertaken to extend the work of these investigators to include New England field soils of varying textural grades and species which had previously not been used as test plants. It is an investigation of the relationship between exudation from detopped plants and soil moisture content.

Materials and methods

Experiments were performed on tomato (*Lycopersicon esculentum* Mill.), tobacco (*Nicotiana tabacum* L.), corn (*Zea mays* L. var. *identata*) and bean (*Phaseolus vulgaris* L.). The soils used were Merrimac Fine Sandy Loam and Suffield Silt obtained from fields on the University Farm, Amherst, Massachusetts. Results of a mechanical analysis by the pipette method (**20**), wilting percentages (determined with wheat plants), organic matter contents, and moisture equivalents are given in table I.

All plants were started in flats and later transplanted to 4-inch, porous clay pots. The plants were never more than 15 cm. tall at the time of transplanting. At the time of experimentation the plants were four to six

TABLE I

MECHANICAL ANALYSIS, ORGANIC MATTER CONTENTS, WILTING PERCENTAGES, AND MOISTURE EQUIVALENTS OF SOILS USED

		Per cent. of dry sample	
		Merrimac fine sandy loam	Suffield silt
Clay	0.002 mm.	9.79	33.85
Silt	0.002–0.05 mm.	49.89	56.72
Fine gravel	retained on 1 mm. sieve	0.05	0.00
Coarse sand	retained on 0.5 mm. sieve	1.02	0.04
Medium sand	retained on 60 mesh sieve	1.14	0.14
Fine sand	retained on 140 mesh sieve	9.04	2.17
Very fine sand	retained on 300 mesh sieve	29.07	7.08
	Total mineral fraction	100.00	100.00
Organic matter		1.91	3.49
Moisture equivalent		17.38	33.82
Wilting percentage		4.50	12.35

weeks old and all were at least 30 cm. tall. From the time of transplanting until actually detopped they were watered occasionally with a complete nutrient solution of low osmotic pressure (approximately 0.5 atmos.). Better root distribution was obtained in Merrimac sandy loam but in both soils roots were generally well distributed throughout the soil mass.

On the evening prior to the day on which determinations of exudation rates were to be made, a group of plants selected at random from those available was brought from the greenhouse to the laboratory. If at this time differences in soil moisture content of individual pots were not apparent, unequal amounts of water were added to the soil of the test plants. This assured a greater range for each set of determinations (**11**). None of the pots was allowed to dry down to a point at which any wilting was evident. The pots were then placed in metal containers, covered with oilcloth to prevent evaporation from the soil surface, and allowed to stand overnight in the laboratory.

The following morning the top of each test plant was removed about four to six cm. above the soil surface with a sharp razor. A graduated 10-ml. pipette was attached to each stump by means of close-fitting rubber tubing and grafting wax. Enough water was placed in each pipette to make a meniscus easily visible. Suction was applied for a short time to remove any air trapped in the stump, roots, or connections. An initial observation of water volume was recorded in milliliters for each pipette. At the end of a 4-hour period a final reading was made.

All experiments were started at approximately 9:30 a.m. It took approximately one hour to set up 15 plants. Therefore exudation was being measured over a period when it is at a maximum. According to GROSSENBACHER (**8, 9**) the period of maximum exudation is at approximately noon. HAGAN (**10**) found minimum negative exudation at this time.

After the 4-hour reading had been recorded, the pipettes were removed and the soil of each pot was sampled for moisture content. A sample of 70 to 80 grams of soil was taken from each pot with a small spatula. As many root fragments as possible were removed. The samples were weighed and oven-dried at 105° C for 48 hours. The moisture content expressed as percentage of dry weight was then calculated.

Results and discussion

The rates of exudation plotted against the corresponding soil moisture content are shown in figures 1, 2, 3, and 4. The parabolic curve was derived by the least square technique (**22**). However, in order to reduce the large number of calculations necessary if each set of values was tabulated individually, averages for both exudation and soil moisture were used in deriving the equations for the eight curves. The equations were differentiated to ascertain the theoretical moisture content at which exudation was a maximum. Equations and theoretical moisture content of maximum exudation for all plants and soil types are given in table II.

TABLE II

RELATIONS BETWEEN SOIL MOISTURE CONTENT AND RATE OF EXUDATION AND AVAILABILITY OF WATER FOR ACTIVE ABSORPTION*

Plant		No. of plants used	No. of grouped headings	Theoretical per cent. moisture at maximum exudation	Per cent. of water in range between W.P. and M.E. which is available to de-topped roots
		Merrimac Fine Sandy Loam			
Bean	$Y = -0.4374 + 0.0408X - 0.00035X^2$	50	14	58.4	42.24
Corn	$Y = -0.7919 + 0.0802X - 0.00084X^2$	55	15	47.9	42.77
Tobacco	$Y = -1.1525 + 0.1262X - 0.00256X^2$	55	16	24.6	41.77
Tomato	$Y = -4.1955 + 0.3533X - 0.00615X^2$	122	20	28.7	4.89
		Suffield Silt			
Bean	$Y = -2.5854 + 0.1376X - 0.00166X^2$	54	13	41.2	19.28
Corn	$Y = -8.0468 + 0.4782X - 0.00632X^2$	54	13	37.8	39.92
Tobacco	$Y = -1.2437 + 0.0456X - 0.00028X^2$	60	17	80.6	1.26
Tomato	$Y = -4.3747 + 0.0194X - 0.00162X^2$	73	14	59.9	16.58

* Y = ml. exudation for 4-hour period and X = soil moisture content on dry weight basis.

Curves for active absorption for various species in both silt and loam are of the same general type. The curves for silt, however, begin at higher soil moisture contents than those of loam because of the higher water-holding capacity of the silt. Figures 1, 2, 3, and 4 indicate that root pressure is not positive throughout the range from moisture equivalent to wilting percentage. As soil moisture content increases the amount of negative exudation decreases until it reaches zero. With a further increase in soil moisture, exudation becomes positive and eventually reaches a maximum.

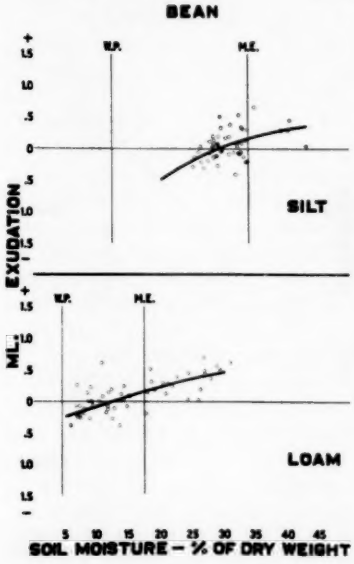


FIG. 1

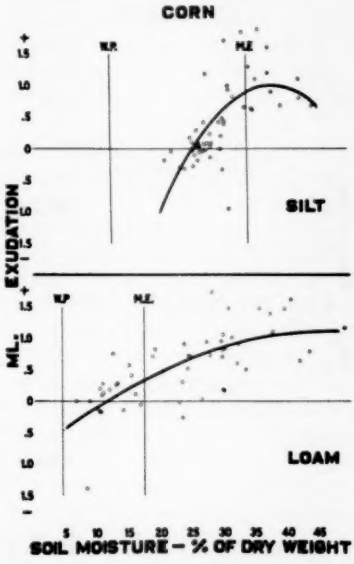


FIG. 2

FIG. 1. Milliliters of exudation plotted against respective soil moisture contents for bean plants in silt and loam.

FIG. 2. Milliliters of exudation plotted against respective soil moisture contents for corn plants in silt and loam.

Maximum positive exudation, however, in all cases occurred when soil moisture content was higher than the moisture equivalent. This observation is not in agreement with the data of McDERMOTT (19) for sunflowers in a sandy loam. McDermott found that a maximum was reached at about the moisture equivalent. Reduction in positive rates of exudation for several of the species at excessively high soil moisture content is probably due to decreased aeration and an increase in the concentration of carbon dioxide around the roots (3, 13). It seems likely that the moisture equivalents of Merrimac Fine Sandy Loam and Suffield Silt are not accurate estimates of

their respective field capacities which are probably higher. Since in every case the maximum rates of exudation were at soil moisture contents in excess of moisture equivalent, apparently aeration was not limiting near the moisture equivalent. This indicates that capillary capacity was not exceeded until moisture content was considerably higher than the moisture equivalent. McDermott's (19) determinations of moisture equivalent were probably a better estimate of the field capacity of his soil, and this observation is supported by the fact that he observed effects of decreased aeration

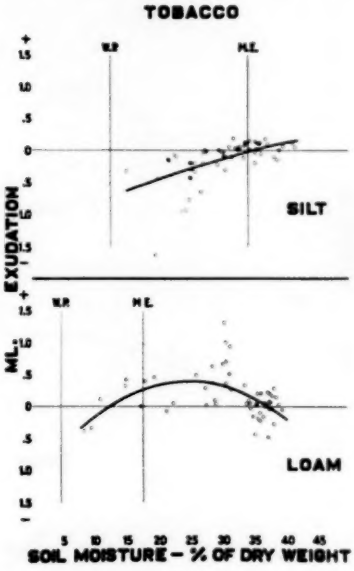


Fig. 3

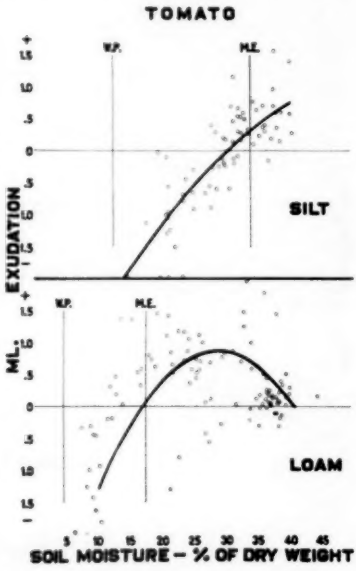


Fig. 4

Fig. 3. Milliliters of exudation plotted against respective soil moisture contents for tobacco plants in silt and loam.

Fig. 4. Milliliters of exudation plotted against respective soil moisture contents for tomato plants in silt and loam.

at about the moisture equivalent. The discrepancies in the actual percentage of water available as reported by Kramer (14) and McDermott (19) may be partly explained by the fact that although moisture equivalent is often used as an estimate of the upper limit of available moisture, it is very often not an adequate estimate of field capacity which is essentially equal to capillary capacity (26). In certain soils field capacity may be twice moisture equivalent (15).

Figures 1, 2, 3, and 4 indicate that root pressure is negligible, or in fact, negative when soil moisture content is in the lower 60% of the range between wilting percentage and moisture equivalent. In sandy loam, with the excep-

tion of tomato, approximately 58% of soil moisture available to the whole plant when the soil is at the moisture equivalent is not available to the detopped root system. In the calculated curve for tomato in loam (fig. 4) the positive slope is increased by the several exceedingly high negative values at high soil moisture content. If the erratic negative values are disregarded, the curve would cross the zero line for exudation at about 11% soil moisture content. This adjusted curve would then give one comparable to those of the other three species for the percentage of soil moisture available to the detopped plant for exudation.

In silt, tobacco showed very little exudation at any moisture content (fig. 3). Tobacco exhibited positive exudation only in the upper 1.26% of the range of moisture between the moisture equivalent and wilting percentage. Corn (fig. 2) actively absorbed water in approximately 40% of the available range.

A total of 60% of soil moisture in the range from wilting percentage to moisture equivalent appears to be the minimum proportion that is not available to detopped root systems. The results of the present study are in general agreement with the findings of Kramer and McDermott in demonstrating that when soil moisture is depleted to the lower part of the available range, active absorption is depressed. Somewhat less moisture was found to be available for active absorption than reported by Kramer and in a few cases less than reported by McDermott. These differences may be due to the fact that moisture equivalent is used to estimate the upper limit of available moisture. The relationship of moisture equivalent to capillary capacity of the different soils used is not clarified although McDermott's data and those of the present study suggest that the degree of reliability concerned is different.

A statistical analysis indicates significant differences in exudation as conditioned by soil types and species. For loam there is a highly significant difference between corn and all other species. Bean shows a significant difference at the 5% level when compared with tomato. The indication is that the observed differences would be due to chance not more than five times out of 100. However, no statistically significant differences were shown to exist between bean and tobacco or tobacco and tomato.

When the same type of analysis of variance is made for plants grown in silt, tobacco when compared with tomato yields no difference of statistical significance. Tobacco did, however, show a statistical difference beyond the 1% level when compared with bean and corn. Corn, bean, and tomato when compared all exhibited highly significant differences in the total amounts of fluid exuded by plants grown in silt.

When a comparison is made for soil types there exists a difference beyond the 1% level for all but corn. Particle size and organic matter content of the soil probably influence the total amount of exudation exhibited by some species. The reasons for the great variation in amounts of water available to four different species in Suffield Silt are not clear. It may be that decreased aeration is a significant factor and a differential species

response is manifested because of hereditary effects on physiological processes of species concerned.

The very small amounts of exudate produced by all species in both Merrimac Fine Sandy Loam and Suffield Silt further demonstrate the inadequacy of active absorption in supplying tops of plants with water. In no case did exudation exceed 2 ml. for four hours and in most tests exudation was less than this small amount or in fact negative. In 4-hour time tests of transpiration and exudation of tomato plants, it was found that the maximum amount exuded represented only 2.07% of the water transpired per gram of oven dry weight of foliage. That much greater forces for water intake are developed in shoots than in roots is further indicated in figure 5.

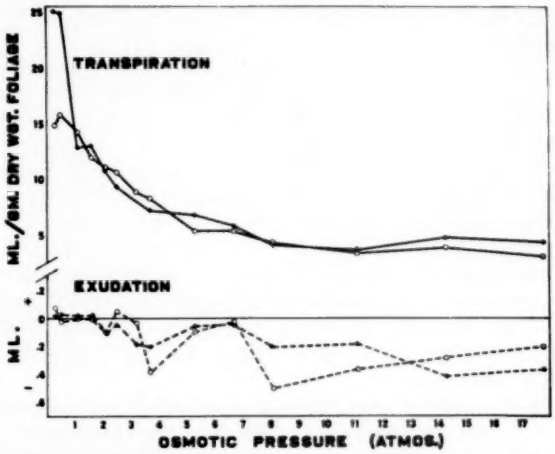


FIG. 5. Transpiration-exudation comparison for tomato plants with roots immersed in sucrose solutions of known osmotic pressure.

Tomato seedlings were grown in sand and watered with a complete nutrient solution. The plants were approximately four weeks old when placed in blackened bottles containing various concentrations of sucrose. Transpiration was determined gravimetrically for a 4-hour period. At the end of this period each bottle was refilled and exudation from stumps of excised plants determined with 10-ml. pipettes in the manner previously described. Figure 5 amplifies the results of two comparisons between transpiration and exudation in sucrose solutions. In neither case was any root pressure manifested when the osmotic pressure of the sucrose solution exceeded three atmospheres and even in solutions of the lowest osmotic pressure the amount of exudate was negligible. The pull developed by intact plants was at least equal to 17.8 atmospheres and this was approximately eight times greater than the maximum force developed by the detopped roots in the weakest concentrations of sucrose.

Summary

1. Relationships between exudation from detopped plants and soil moisture content were studied for Merrimac Fine Sandy Loam and Suffield Silt. Species used as test plants were tomato (*Lycopersicum esculentum* Mill.), tobacco (*Nicotiana tabacum* L.) corn (*Zea mays* L. var. *identata*), and bean (*Phaseolus vulgaris* L.).

2. A parabolic relationship was found between exudation and soil moisture content on a dry weight basis.

3. Soil types yielded significant differences for most species tested when total exudation was compared for silt and loam.

4. A total of approximately 60% of soil moisture in the range from wilting percentage to moisture equivalent appeared to be a minimum value which was not available to detopped root systems.

5. Data are presented which indicate that moisture equivalent may be grossly inadequate as an estimate of capillary capacity of soil.

6. Evidence is presented to show the inadequacy of active absorption in supplying tops of plants with water.

The authors are indebted to Mr. H. M. Yegian of the University of Massachusetts Agronomy Department for counsel on statistical analysis of the data.

DEPARTMENT OF BOTANY
UNIVERSITY OF MASSACHUSETTS
AMHERST, MASSACHUSETTS

LITERATURE CITED

1. ALDRICH, W. W. and WORK, R. A. Evaporating power of the air and top–root ratio in relation to rate of pear fruit enlargement. Proc. Amer. Soc. Hort. Sci. **32**: 115–123. 1934.
2. AYERS, A. D., WADLEIGH, C. H., and MAGISTAD, O. C. The interrelationships of salt concentration and soil moisture content with the growth of beans. Jour. Amer. Soc. Agron. **35**: 796–810. 1943.
3. CHANG, H. T. and LOOMIS, W. E. Effect of CO_2 on absorption of water and nutrients by roots. Plant Physiol. **20**: 221–232. 1945.
4. DAVIS, C. H. Absorption of soil moisture by maize roots. Bot. Gaz. **101**: 791–805. 1940.
5. DOREEN, L. D. and MACGILLIVRAY, J. H. Germination (emergence) of vegetable seed as affected by different soil moisture conditions. Plant Physiol. **18**: 524–529. 1943.
6. FURR, J. R. and REEVE, J. O. The range of soil-moisture percentages through which plants undergo permanent wilting in some soils from semiarid irrigated areas. Jour. Agr. Res. **71**: 149–170. 1945.
7. FURR, J. R. and TAYLOR, C. A. Growth of lemon fruits in relation to moisture content of the soil. U. S. Dept. Agr. Tech. Bull. **640**. 1939.

8. Grossenbacher, K. A. Diurnal fluctuations in root pressure. Plant Physiol. **13**: 669–676. 1938.
9. Grossenbacher, K. A. Autonomic cycle of rate of exudation of plants. Amer. Jour. Bot. **26**: 107–109. 1939.
10. Hagan, R. M. Autonomic diurnal cycles in the water relations of non-exuding detopped root systems. Plant Physiol. **24**: 441–453. 1949.
11. Hendrickson, A. H. and Veihmeyer, F. J. Moisture distribution in soil in containers. Plant Physiol. **16**: 821–826. 1941.
12. Kozlowski, T. T. Light and water in relation to growth and competition of Piedmont Forest tree species. Ecol. Monog. **19**: 207–231. 1949.
13. Kramer, P. J. Causes of decreased absorption of water by plants in poorly aerated media. Amer. Jour. Bot. **27**: 216–220. 1940.
14. Kramer, P. J. Soil moisture as a limiting factor for active absorption and root pressure. Amer. Jour. Bot. **28**: 446–451. 1941.
15. Kramer, P. J. Plant and soil water relationships. McGraw-Hill, New York. 1949.
16. Lewis, M. R., Work, R. A., and Aldrich, W. W. Influence of different quantities of moisture in a heavy soil on rate of growth of pears. Plant Physiol. **10**: 309–323. 1939.
17. Litvinov, L. S. and Gebhardt, A. G. Bleeding of Steppe plants. Bull. Inst. Rech. Biol. Univ. Perm. **6**: 91–111. In Russian. Biol. Abstracts **3**: 17877. 1929.
18. Magness, J. R., Degman, E. S., and Furr, J. R. Soil moisture and irrigation investigations in eastern apple orchards. U. S. Dept. Agr. Tech. Bull. 491. 1935.
19. McDermott, J. J. The effect of the moisture content of the soil upon the rate of exudation. Amer. Jour. Bot. **32**: 570–574. 1945.
20. Olmstead, L. B., Alexander, L. T., and Middleton, H. E. A pipette method of mechanical analysis of soils based on improved dispersion procedure. U. S. Dept. Agr. Tech. Bull. 170. 1930.
21. Schopmeyer, C. S. Transpiration and physico-chemical properties of leaves as related to drought resistance in loblolly pine and shortleaf pine. Plant Physiol. **14**: 447–462. 1939.
22. Snedecor, G. W. Statistical methods. Iowa State College Press, Ames, Iowa. 1940.
23. Veihmeyer, F. J. and Hendrickson, A. H. Soil moisture conditions in relation to plant growth. Plant Physiol. **2**: 71–82. 1927.
24. Veihmeyer, F. J. and Hendrickson, A. H. Soil moisture in relation to plant growth. Ann. Rev. Plant Physiol. **1**: 285–304. 1950.
25. Wadleigh, C. H. and Ayers, A. D. Growth and biochemical composition of bean plants as conditioned by soil moisture. Plant Physiol. **20**: 106–132. 1945.
26. Work, R. A. and Lewis, M. R. Moisture equivalent, field capacity and permanent wilting percentage and their ratios in heavy soils. Agr. Eng. **15**: 1–20. 1934.

EFFECTS OF FLOODED SOIL ON GROWTH OF PINE SEEDLINGS

FRANCIS M. HUNT

Received June 23, 1950

Introduction

Most crop and horticultural plants are seriously injured or even killed if the soil in which they are growing is flooded with water for an extended period of time. It generally is assumed that flooding is equally injurious to most species of forest trees and limits the natural distribution of many species to relatively well drained sites. It is supposed, for example, that pond pine occupies wet sites in the Atlantic Coastal Plain because its roots can survive flooding and the accompanying poor aeration better than the roots of loblolly pine or shortleaf pine.

This view was not supported by the results of an experiment by GAISER (1) who studied the effects of flooding on loblolly pine and pond pine seedlings grown in the greenhouse. The seedlings were planted in 5-quart oil cans filled with sandy soil. Holes were punched in the bottom of the cans and a layer of gravel was placed beneath the soil to facilitate drainage of the soil. Various periods of flooding, alternating with periods during which the soil was drained, were maintained for three months in the spring of 1948. The loblolly pine seedlings appeared to be more resistant to flooding injury than pond pine seedlings, and both species made better growth in soil intermittently flooded for short periods (two days out of nine) than in soil maintained at field capacity or lower. These results were so unexpected that a more extensive experiment was planned to study the effects of flooding.

Methods and design of the experiment

The three species of pine used in this experiment were shortleaf pine (*Pinus echinata* Mill.), loblolly pine (*Pinus taeda* L.), and pond pine (*Pinus rigida* var. *serotina* (Michx.) Loud.). The shortleaf pine and loblolly pine seedlings were 1–0 stock, obtained from the state nursery at Clayton, N. C. The pond pine seedlings were 1–1 stock obtained from the Bladen Lakes State Forest where they had been planted as 1–0 nursery stock the preceding spring.

Three seedlings, one of each of the three species, were planted in each of 48 cans. These were 5-quart oil cans which had been given an interior coating of asphalt, and were filled with sandy soil underlain by a layer of gravel to provide drainage. The soil used is known as Lewisburg sand and resembles Norfolk sand which is common in the Atlantic Coastal Plain. In 12 of the cans a glass tube was placed along one side, extending into the gravel so that water could be removed from the bottom of the can by a constant level siphon. In this manner well aerated water could be circulated.

After planting the seedlings about December 1, 1948, the cans were placed in the greenhouse and maintained at field capacity until the treatments were started on February 8, 1949. At this time the seedlings were just breaking dormancy and appeared to be healthy and free from disease.

The original experiment consisted of four treatments, each containing 12 cans with one seedling of each species in every can. After three months each treatment was subdivided and half of the seedlings were subjected to a cycle of drying and wetting as described under treatment A–2. This was done because it was believed that even though the roots had been injured by flooding they might absorb enough water from saturated soil for successful growth, but this would be impossible in drained soil.

The treatments were as follows:

A–1. Flooding with moving water. Water was supplied by a 5-gallon reservoir, equipped with a constant-level intake valve, which was suspended above the cans. The water in the reservoir tank was aerated for about three hours each week by bubbling air through it. A tube ran from the reservoir to each of the twelve cans and the rate of water drip was controlled by a screw clamp. Excess water was removed from the flasks by constant-level siphons placed in the glass tube mentioned earlier. The water in the flasks was returned twice daily to the reservoir to eliminate the effects of leaching of minerals by the moving water.

A–2. At the end of three months, or about May 1, six of the cans in A–1 were drained to field capacity and allowed to dry down toward permanent wilting. When the amount of water lost daily became negligible the soil moisture was brought back to field capacity. This cycle was continued for the remaining seven months of the experiment.

B–1. Alternate flooding. The 12 cans in this experiment were flooded for two weeks and then drained by a vacuum pump, and maintained at field capacity for two weeks after which they were again flooded. This 4-week cycle was continued throughout the experiment.

B–2. Six of the cans from B–1 were treated as in A–2 above after May 1.

C–1. Continual flooding. These 12 cans were kept flooded with stagnant water to a level slightly above the soil surface during the entire experiment.

C–2. Six of the cans from C–1 were treated the same as A–2 above after May 1.

D–1. Controls. The 12 control cans were maintained at or near field capacity for the entire experiment.

D–2. Six of the cans from D–1 were treated the same as A–2 above after May 1.

The cans were arranged as compactly as possible in a rectangle on the greenhouse floor and were exposed to an essentially identical environment.

Every two weeks a record was made of the individuals which had died, the increase or decrease in the number of needles, the height growth, and the number of side branches gained or lost. At the end of the experiment a statistical analysis was made on those portions of the data, the significance of which was not otherwise obvious. The general results of this statistical analysis are given in the Discussion section.

Results

The results of these experiments are summarized in the following tables:

TABLE I

HEIGHT GROWTH OF PINE SEEDLINGS GROWN UNDER VARIOUS CONDITIONS. PERIOD ONE REPRESENTS THE INITIAL 12-WEEKS' TREATMENT. EACH GROUP WAS THEN DIVIDED AND GROUPS A-2, B-2, C-2, AND D-2 WERE DRIED DOWN TO APPROXIMATELY THE PERMANENT WILTING PERCENTAGE BEFORE WATERING. THIS CYCLE OF WETTING AND DRYING WAS MAINTAINED FOR THE REMAINING SEVEN MONTHS OF THE EXPERIMENT AND THE GROWTH IS INDICATED AS PERIOD 2.

Treatment		Shortleaf pine Period		Pond pine Period		Loblolly pine Period	
		1	2	1	2	1	2
A-1	Flooded with moving water	4.02*	0.83	2.00	2.30	3.80	1.55
A-2		...	0.15	...	0.65	...	1.23
B-1	Periodically flooded	3.78	1.05	2.40	0.95	4.38	1.37
B-2		...	0.50	...	0.54	...	1.36
C-1	Flooded with standing water	2.31	1.07	1.15	0.20	4.12	0.98
C-2		...	1.30	...	0.30	...	2.60
D-1	Kept at field capacity	4.63	0.95	2.25	2.16	5.09	1.14
D-2		...	0.46	...	0.37	...	1.95

*Amount of growth is expressed in cm.

TABLE II

NUMBER OF SEEDLINGS WHICH DIED WITH EACH TREATMENT. (LEGEND OF TREATMENT SAME AS TABLE I)

Treatment	Shortleaf pine Period		Pond pine Period		Loblolly pine Period		Dry weight of root systems*
	1	2	1	2	1	2	Grams
A-1	0	1	2	1	0	1	1.897
A-2	...	2	...	1	...	1	1.206
B-1	0	1	4	4	0	1	1.705
B-2	...	2	...	1	...	1	1.372
C-1	1	1	2	1	0	1	1.256
C-2	...	2	...	1	...	2	1.432
D-1	0	1	1	1	0	1	1.953
D-2	...	2	...	2	...	2	1.413

*Average weight at end of experiment by treatments.

Condition of the different groups of roots when removed from the containers:

A–1. New roots were generally found on all plants of all species; however, the older roots appeared dark in color and many were dead.

B–1. No growing root tips, roots blackish brown in color and many dead.

C–1. No growing root tips found and most older roots appeared dead.

D–1. Normal root systems, light brown in color, with growing root tips.

A–2, B–2, C–2, D–2 all appeared to have normal root systems with light brown color and growing root tips.

Discussion

It was shown by the analysis of the data that during the first 12 weeks the trees in soil maintained near field capacity made more height growth than the trees flooded with standing water, but the other treatments did not significantly reduce growth. Loblolly pine and shortleaf pine seedlings made more growth than pond pine seedlings, regardless of treatment, and the greatest number of deaths occurred among pond pine seedlings. There were no significant differences in needle growth or in increase in number of branches.

The analysis of the data further showed that after another seven months differences between treatments disappeared where the original treatments were continued. Among the seedlings changed to the drying cycle those previously flooded with moving water made less growth than those which had previously received other treatments. Loblolly pine made more height growth than the other species when changed to the drying cycle. No differences in death rate occurred among the trees changed to the drying cycle. The roots of the continuously flooded group weighed less than those of other groups, but no significant differences in dry weight were found among the trees changed to the drying cycle.

It was surprising to find so little injury resulting from flooding the soil. The only treatment which reduced shoot growth appreciably during the first three months was continuous flooding with standing water, and even the effect of this treatment disappeared during the succeeding seven months. Dry weight of root systems was reduced by this treatment, however, and the roots were in such poor condition that it seems probable that, if the experiment had been continued into a second growing season, serious reduction in growth or even death of the seedlings would have occurred. PARKER (3) also found that loblolly pine can survive for a year or more in flooded soil, even though the root systems appeared to be dead. Perhaps such survival is possible because transpiring plants can absorb water through dead roots so long as they do not disintegrate (KRAMER, 2). Flooding with running water would be expected to produce less injury than standing water because moving water should supply more oxygen and prevent accumuluation of carbon dioxide or other toxic substances. Although the shoots showed no injury, the root systems were somewhat injured in the containers continu-

ously flooded with flowing water, though less so than those in containers flooded two weeks out of every four. It is probable that although the surplus water was drained off of these plants every two weeks, the soil was not actually reduced to field capacity. In spite of the injury to the roots from periodic flooding, the tops showed no injury. This is in line with the observation of GAISER (1) that both pond pine and loblolly pine made better shoot growth in soil flooded two days out of nine than in soil kept continuously at field capacity. One of the most surprising results of this experiment was the fact that, although observable injury to root systems was produced by flooding, little injury to the shoots resulted, even when the soil was allowed to dry out. This is directly contrary to experience with herbaceous species and perhaps is partly related to the ability of pine seedlings to survive even if inadequately supplied with water.

The failure of pond pine to survive better than the other species in flooded soil is surprising since it is supposed to grow well on wet sites but it is in agreement with the results of Gaiser, mentioned previously. Possibly the slightly higher death rate of the pond pine trees might be related to the fact that they were two years old and did not transplant as well as the one-year-old loblolly pine and shortleaf pine. It was assumed that any injury to root systems would be accentuated by drying out the soil. Even though the roots were largely dead they might function as absorbing surfaces as long as the soil was saturated but when it was dried down dead roots would be ineffective. Contrary to expectation, the seedlings which had been flooded for three months were no more affected by repeatedly allowing the soil to dry out than were the seedlings previously kept at field capacity. Apparently flooding for three months does not permanently injure pine root systems. The appearance of the roots after 10 months of flooding suggests that they were now so badly injured that the plants would probably have been killed if the soil had been dried down below field capacity.

The limited mortality and relatively small reduction in shoot growth caused by flooding are surprising and it appears that further work ought to be done on this problem.

Summary

An experiment was conducted to determine the effects of various degrees and methods of flooding on seedlings of shortleaf pine, loblolly pine, and pond pine growing in soil. One group of trees was flooded continuously with standing water, another group with flowing water. A third group was alternately flooded for two weeks and kept at field capacity for two weeks while the control group was kept approximately at field capacity.

The experiment was started in February, and after 12 weeks the only effect was slightly reduced growth of the seedlings continuously flooded with stagnant water. After 12 weeks, the soil in half of the containers from each treatment was allowed to dry down to permanent wilting, then re-watered

to field capacity. This cycle was repeated for seven months with little effect except that those seedlings flooded with running water during the first twelve weeks made less growth than the other groups. The plants continuously flooded with standing water had a lower dry weight than those receiving other treatments. No significant difference in mortality occurred.

In general these pine seedlings proved unusually resistant to injury by flooding.

The author wishes to thank Dr. P. J. Kramer of the Department of Botany, Duke University, for suggesting the problem and for his helpful criticism throughout the work.

KENTUCKY DIVISION OF FORESTRY
SOMERSET, KENTUCKY

LITERATURE CITED

1. GAISER, R. N. The growth of loblolly and pond pine seedlings under differing conditions of soil flooding. Unpublished manuscript. Duke University. 1947.
2. KRAMER, P. J. The intake of water through dead root systems and its relation to the problem of absorption by transpiring plants. Amer. Jour. Bot. **20**: 481–492. 1933.
3. PARKER, JOHNSON. The effects of flooding on the transpiration and survival of some southeastern forest tree species. Plant Physiol. **25**: 453–460. 1950.

THE RELATIONSHIP OF STRUCTURE AND PLANT-GROWTH ACTIVITY OF SUBSTITUTED BENZOIC AND PHENOXYACETIC ACIDS

ROBERT M. MUIR AND CORWIN HANSCH

Received January 7, 1951

Introduction

In earlier papers it was suggested that plant growth-regulators require a free or potentially free ortho position in the unsaturated ring to be effective in promoting cell elongation (**5, 8**) in addition to those structural features previously suggested by KOEPFLI, THIMANN, and WENT (**7**). BENTLEY (**1**), however, has found that 2,3,6-trichlorobenzoic acid and its aldehyde have high activity in promoting cell elongation. Such activity in compounds with both ortho positions occupied by halogen atoms would seem to indicate that the benzoic acids do not enter into a reaction with a plant substrate through the ortho position in the unsaturated ring as postulated for phenylacetic, phenylbutyric, phenoxyacetic and indoleacetic acids (**5, 8**). A study of the elongation effects of a selected series of benzoic acids has been made and the evidence is interpreted to indicate a reaction taking place through an ortho position in the unsaturated ring but involving the displacement of a halogen atom rather than a hydrogen atom.

Materials and methods

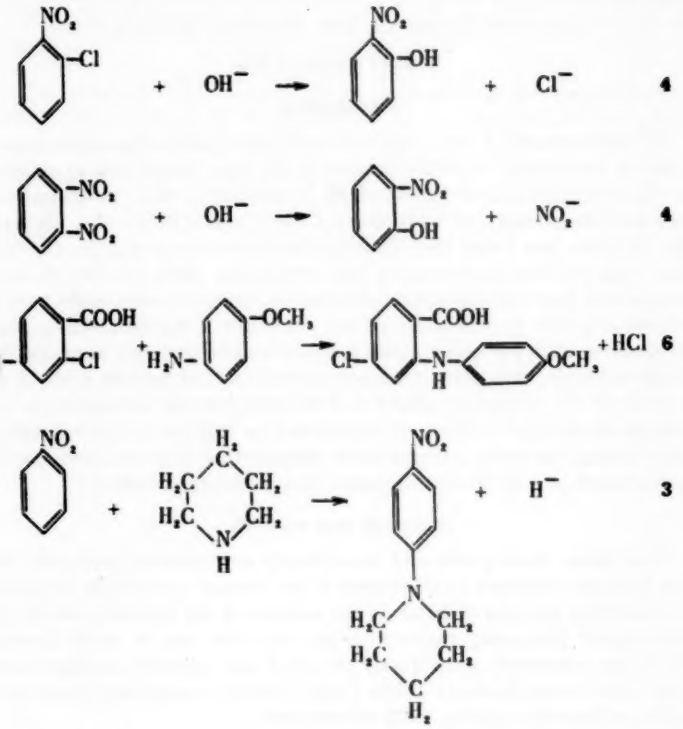
Test plants were grown and used exactly as described previously (**8**). The data are expressed as differences in the average percentage elongation of 15 sections growing in 25 ml. of the solution of the chemical and in distilled water: thus comparisons with previous tests can be made directly. All of the compounds were freshly prepared and carefully purified except a few which were Eastman White Label products crystallized from water until a satisfactory melting point was obtained.

Experimental results and discussion

The data of representative tests of the activities of the benzoic acids at various concentrations are found in table I. Benzoic acid has no growth-promoting activity nor does any derivative in which the ortho position is not substituted with an electronegative group capable of displacement by an electron-rich substrate. Benzoic acids with substituents only in the meta or para positions are not active. Thus, if a reaction takes place at an ortho position, it would be possible only by displacement of the electron-attracting group (halogen or nitro group) from this position. This is not unreasonable in view of the fact that electronegative groups ortho and para to a halogen or nitro group on a benzene ring promote displacement of such groups by nucleophilic groups. Such reactions are well known in organic

chemistry as is illustrated by the following examples (a general discussion of this type of reaction is given by REMICK, 9).

Reference



Thus we might write the reaction for 2,3,6-trichlorobenzoic acid with an electron-rich plant substrate X^- as follows:

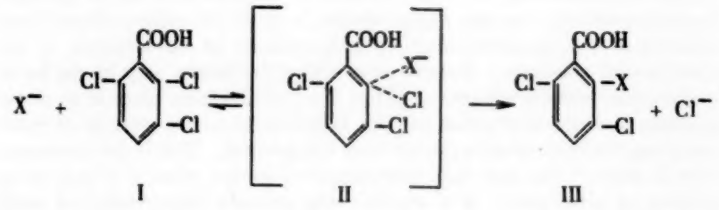


TABLE I

PERCENTAGE ELONGATION OF SECTIONS OF COLEOPTILES IN SOLUTIONS OF BENZOIC ACIDS AS COMPARED WITH PERCENTAGE ELONGATION IN WATER

Compound	Molar concentrations × 10^{-4}								
	5.0	3.0	2.0	1.0	0.5	0.1	0.05	0.01	0.005
Benzoic acid	−13	0	0	0	0	0		0	
o-Chlorobenzoic acid	−19		+10	+ 8	+ 6	0	0	0	
m-Chlorobenzoic acid			−19	− 5	0	0	0	0	
p-Chlorobenzoic acid			−17	− 8	0	0	0	0	
o-Bromobenzoic acid			−11	+ 4	+ 8	0		0	
m-Bromobenzoic acid			−16	−11	0	0		0	
p-Bromobenzoic acid			−15	−11	0	0			
o-Iodobenzoic acid			−11	+ 8	+ 4	0		0	
m-Iodobenzoic acid			−17	− 9	0	0		0	
p-Iodobenzoic acid				− 6	− 5	0	0		
o-Nitrobenzoic acid	−12		+ 9	+ 6	+ 3	0			
m-Nitrobenzoic acid	−20		− 9	0	0	0		0	
p-Nitrobenzoic acid	−14		−10	0	0	0			
o-Hydroxybenzoic acid		−18	−12	0	0	0			
m-Hydroxybenzoic acid	0	0	0	0	0	0			
p-Hydroxybenzoic acid	0	0	0	0	0	0			
o-Aminobenzoic acid	0	0	0	0	0	0			
p-Aminobenzoic acid	0		0	0	0	0			
2,4-Dichlorobenzoic acid				−12	− 8	0	0	0	
2,5-Dichlorobenzoic acid			−10	+ 9	+19	+11	+ 6	0	
2,6-Dichlorobenzoic acid		+ 5	+18	+17	+ 6	0		0	
2,3,5-Triiodobenzoic acid					+ 7	+12	+15	+13	+10
2-Amino-3,5-Diiodobenzoic acid				−19	−10	0	0	0	0
2-Chloro-5-Nitrobenzoic acid			0	0	0	0	0	0	
2,6-Dimethoxybenzoic acid	−10		0	0	0	0		0	
2,4,6-Trimethylbenzoic acid*			− 3	0	0	0	0	0	
2,4,6-Triisopropylbenzoic acid*				0	0	0		0	

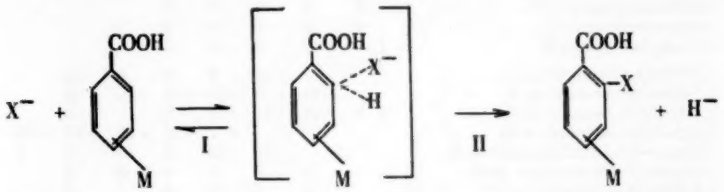
*These compounds were obtained through the courtesy of Dr. M. S. Newman of Ohio State University.

This mechanism is possible for the benzoic acids because of the electron-attracting character of the carboxyl group which promotes the formation of the intermediate II which can then decompose to give product III. However, such a mechanism is not possible in the phenoxyacetic acid series because of the higher electron density in the benzene ring due to the electron-rich nature of the phenoxy linkage. Displacement of electronegative groups from the phenoxyacetic acids would not be promoted by such a system; thus 2,6-dibromophenoxyacetic acid is not active (**5**) while 2,6-dichlorobenzoic acid is. Further indication that the reaction involves the removal of an electronegative group of the ortho position is found when one examines the activities of compounds in which the ortho positions are occupied by electron-rich groups, *i.e.*, amino and hydroxyl groups which are not capable of displacement. From table I it is seen that both *o*-aminobenzoic and *o*-hydroxybenzoic acids are inactive. Furthermore, although two halogen atoms are present in the ring, the lack of an electronegative group in the ortho position gives a compound without activity, as illustrated by 2-amino-3,5-diiodobenzoic acid.

When electron-releasing groups (instead of electronegative groups) are substituted in both ortho positions in benzoic acid as with 2,6-dimethoxybenzoic acid, 2,4,6-trimethylbenzoic acid and 2,4,6-triisopropylbenzoic acid the resulting compound is inactive. This is to be expected since these electron-releasing groups would not be capable of displacement by an electron-rich plant substrate and thus would block reaction at the ortho position.

This general concept is also supported by the observations of WEINTRAUB and NORMAN (11). These investigators note that halogen and nitro groups (electronegative groups) increase the activity of phenylacetic and benzoic acids while methyl, hydroxyl and amino groups (electron-rich groups) diminish the activity when introduced into benzoic acids or phenylacetic acids.

The above discussion does not mean that all benzoic acids must have an electronegative group in an ortho position to be active. This may be seen by a consideration of the following general equation for the reaction of a substituted benzoic acid where M represents one or more substituents and X^- represents a hypothetical nucleophilic plant substrate.

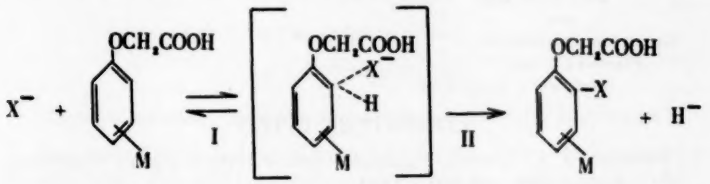


Two important factors determine whether the reactions will take place at an appreciable rate. First, the more electron-attracting M is, the more tendency the transition-complex will have to form. However, the more electron-attracting M is, the less tendency there will be for reaction II to take place with the displacement of H^- (probably brought about by an electron-deficient oxidizing system). Thus a balance between the two effects in the molecule is necessary for both steps to take place at a sufficient rate for the substance to display activity as a growth-regulator. This condition would explain also why a chlorine atom or a nitro group in an ortho position would permit reaction by relatively easy displacement as Cl^- or NO_2^-. Thus a benzoic acid in which the carboxyl group exerts a strong enough electronic pull to initiate the formation of complex and the combined electronic attraction of the carboxyl and M is not too great to permit the removal of H^- would show activity without having an electronegative group in the ortho position.

This theory has been developed as an explanation of the activity of both the benzoic acids and the phenoxyacetic acids in bringing about cell elongation. A reaction with a nucleophilic plant substrate is postulated because only those phenoxyacetic acids which are substituted with electron-attract-

ing groups show appreciable cell elongation activity (**8, 11**). This theory also seems to be the best way in which to rationalize opposite effects, namely, that in benzoic acid negative groups in both ortho positions result in an active compound, while in the phenoxyacetic acids negative groups in both ortho positions result in a complete loss of activity.

The conditions operative for the reaction of a hypothetical nucleophilic substrate with a phenoxyacetic acid are the same as for the benzoic acids.



In this series of compounds the rate-controlling step would be reaction I probably and unless M has enough electron-attracting power to induce this reaction, then the overall reaction will not take place. For the benzoic acids step II might be the one controlling the rate rather than I. In the case of the phenoxyacetic acids reaction II would take place relatively easily because of the electron-releasing character of the phenoxy linkage.

In view of the relationship of structure and plant-growth activity demonstrated by the series of substituted benzoic acids the minimum characteristics required for a compound to be active as a growth-regulator may be described as follows: 1) An unsaturated ring (preferably aromatic) must be present (**7**). 2) A carboxyl group or a group capable of conversion to a carboxyl group such as an ester or a nitrile must be present. This group may be attached through a chain of one or more atoms (**7**) or directly to the unsaturated ring. 3) At least one of the positions on the ring ortho to the point of attachment of the carboxyl group or the side chain carrying the carboxyl group must be capable of reaction with an electron-rich plant substrate. The ortho position must hold the proper electron density and the group or atom at that position (be it H, Cl, NO_2, etc.) must be capable of displacement under the conditions of reaction. 4) According to VELDSTRA and BOOIJ (**10**) the relative lipophilic character of the molecule compared to the hydrophilic nature of certain attached groups such as the carboxyl group may affect the activity of a compound as a growth regulator. 5) Characteristics such as the relation of the side chain carboxyl group to an ortho position (**2**) may be of importance in determining the activity of a compound but little is known about them.

Summary

In a series of substituted benzoic acids only those with an electronegative atom or group capable of displacement by an electron-rich substrate in

one or both ortho positions can promote cell elongation of Avena coleoptile sections.

A mechanism of reaction of the growth regulator with the plant substrate based upon electronic characteristics of active benzoic acids and phenoxyacetic acids is described.

BOTANY DEPARTMENT
STATE UNIVERSITY OF IOWA
IOWA CITY, IOWA
AND
CHEMISTRY DEPARTMENT
POMONA COLLEGE
CLAREMONT, CALIFORNIA

LITERATURE CITED

1. BENTLEY, J. A. Growth-regulating effect of certain organic compounds. Nature **165**: 449–450. 1950.
2. BOOIJ, H. L. and VELDSTRA, H. Researches on plant growth regulators. XVI. The effect of plant growth substances on coacervates. Biochim. Biophys. Acta. **3**: 260–277. 1949.
3. BRADLEY, W. and ROBINSON, R. Kationoid reactivity of aromatic compounds. Jour. Chem. Soc. **1932**(1): 1254–1263.
4. FIESER, L. F. and FIESER, M. Organic chemistry. 2nd ed. pp. 611–612. D. C. Heath, New York. 1950.
5. HANSCH, C. and MUIR, R. M. The ortho effect in plant growth-regulators. Plant Physiol. **25**: 389–393. 1950.
6. JENKINS, G. L. and HARTUNG, W. H. Chemistry of organic medicinal products. 3rd ed. p. 563. Wiley, New York. 1949.
7. KOEPFLI, J. B., THIMANN, K. V., and WENT, F. W. Phytohormones: Structure and physiological activity. I. Jour. Biol. Chem. **122**: 763–780. 1937–1938.
8. MUIR, R. M., HANSCH, C. H., and GALLUP, A. H. Growth regulation by organic compounds. Plant Physiol. **24**: 359–366. 1949.
9. REMICK, A. E. Electronic interpretations of organic chemistry. 2nd ed. pp. 374–377. Wiley, New York. 1949.
10. VELDSTRA, H. and BOOIJ, H. L. Researches on plant growth regulators. XVII. Structure and activity. On the mechanism of action. III. Biochim. Biophys. Acta. **3**: 278–312. 1949.
11. WEINTRAUB, R. L. and NORMAN, A. G. Plant growth-regulators. Econ. Bot. **3**: 289–298. 1949.

INVESTIGATIONS ON NITROGEN COMPOUNDS AND NITROGEN METABOLISM IN PLANTS. I. THE REACTION OF NITROGEN COMPOUNDS WITH NINHYDRIN ON PAPER: A QUANTITATIVE PROCEDURE

JOHN F. THOMPSON,[1] R. M. ZACHARIUS[1] AND F. C. STEWARD[1]

(WITH SEVEN FIGURES)

Received October 24, 1950

General introduction to the investigation of nitrogen metabolism

This is the first of a group of papers which will record investigations on nitrogen compounds and their metabolism in plants. The work was undertaken not only because of the intrinsic interest of nitrogen metabolism, but even more because it had become so apparent that other vital phenomena would only be fully comprehended when their relations to nitrogen metabolism were revealed (**26**, and refs. there cited; **27**). Plant respiration—long considered in terms only of carbohydrate breakdown—has recently been shown to include a moiety which varies concomitantly with protein synthesis and the metabolism of amino acids. This was especially true of the cells of the potato tuber (**24**), under the conditions which promote accumulation of salts. In another series of papers (for references see **21**), the ability to accumulate salts, now somewhat generally conceded to be a property of active cells and dependent upon their aerobic respiration, has also been associated in a variety of ways with cells able to grow and to synthesize protein and with factors able to foster this process (**22, 23, 24**). For the particular case of the potato tuber, it was recognized that aerobic respiration, protein synthesis from the stored reserves of soluble nitrogen and the accumulation of anions (Br) and cations (K) varied *pari passu* under the influence of a wide range of conditions. Observations such as these led to the view that the energy of respiration was made available simultaneously for both salt accumulation and the protein synthesis with which it appeared to be linked. The necessary point of contact between the energy derived from aerobic respiration with the metabolism of nitrogen compounds and salt accumulation, promised, therefore, to be fundamental to the understanding of all of these processes.

Prior to the work now to be described, some progress had been made by analytical methods which could fractionate the soluble nitrogen of the cells only into the amino-N and amide-N fractions (**22**). The total amide-N was further separable into glutamine-amide-N and asparagine-amide-N (**25**), and it became apparent that these two substances stood in quite different relationships to protein synthesis (**26**, and refs. there cited). Further-

[1] Present address: Botany Dept., Cornell University, Ithaca, N. Y.

more, it was possible to see in the effects of various salts and oxygen evidence that emphasized the need to locate the point in the nitrogen metabolism at which these variables exerted their several effects.

Prior to the advent of partition chromatography, whether on paper or on columns, methods were not available for separating the amino-N moiety of the soluble nitrogen into its constituent substances, and the existing methods were quite unequal to the task of routine quantitative determinations of the individual components of this fraction.

With the development of the method of partition chromatography on paper by CONSDEN, GORDON, and MARTIN (**5**), a new technique became available for the investigation of amino compounds, and their role in metabolism. In the first applications of the new technique to biological problems, free amino acids were detected and identified in a variety of fluids or extracts, especially blood and urines by DENT (**7**). A systematic application of the procedure to a plant extract awaited the work of DENT, STEPKA, and STEWARD (**8**) who, by its aid, identified some 22 known amino acids and amides in an alcoholic extract of potato tuber, thus adding to the free amino acids known to occur in that tissue: there were, however, in addition several other substances the identity of which was not then known. From this point, a number of workers have used the paper chromatographic procedure to investigate amino acids in various plants: the current trends and the potentialities of this work have been reviewed by STEWARD and THOMPSON (**27**). It is the purpose of this group of papers to present the new experimental evidence bearing upon nitrogen metabolism and cognate problems which has accrued from the application of methods based on paper partition chromatography.

The full exploitation of the paper chromatographic separation of the amino acids requires that it should become the basis of a reliable quantitative procedure for amino acid determinations. Such a method would permit one to trace, simultaneously, the behavior of all the principal amino acids in the tissues and even of some of those that may be present only in very small amount. Such a procedure would be applicable to a variety of nutritional and metabolic investigations.

In the now familiar partition chromatographic method on paper, each amino acid forms a discrete spot which is revealed when the paper is sprayed with a ninhydrin solution. Despite a variety of attempts to make this procedure quantitative (see refs. cited below), our experience was that the many variables inherent in the ninhydrin reaction, as it is carried out on paper, and the many variables in the chromatography needed first to be recognized and, secondly, to be brought under control. It is our purpose to set out in some detail, in this and a succeeding paper, how this has been done and, on the basis of the information so obtained, to describe a rigorous quantitative procedure for the amino acids of plants based on paper chromatography. This is the necessary prelude to the presentation of the new results on nitrogen metabolism which have been obtained.

Quantitative applications of paper chromatography to amino acids

One of the two major problems in developing a method for quantitative determination of amino acids by paper partition chromatography is the measurement of the amino acids after they have been chromatographed. A number of procedures have been proposed which include colorimetric and polarographic methods as well as the use of a photoelectric densitometer.

POLSON *et al.* (**18**) were among the first to report a quantitative procedure for determining amino acids after separation by paper partition chromatography. This technique included the development of a color with amino compounds and ninhydrin on the paper, the extraction of the color with acetone and the measurement of the color. Aside from the fact that acetone is a poor solvent for the colored product, these authors apparently found other disadvantages to this procedure because it was abandoned for one (**17**) in which visual comparisons were made with standard amounts of amino acids chromatographed simultaneously with unknowns. This technique has the disadvantage that it requires a large number of standards with each sample, or set of samples, and it depends on a subjective test which can only be approximately quantitative.

Several subsequent methods have utilized 1-directional chromatography, formed a color with ninhydrin, and measured the color by the density or size of colored areas. FISHER *et al.* (**9**) measured the area of the spot, formed by the ninhydrin reaction, and found that the size was proportional to the logarithm of the concentration of the amino acid. The concentration of an amino acid in a sample can then be determined by comparison with standards run simultaneously. One technique they used was a photographic method for determining spot size. BULL *et al.* (**4**) determined the light transmission of the colored compound on the paper at different points along the spot and in the direction of phenol movement. By integrating the area under the curve relating color density and distance, an estimate of quantity was made. BLOCK (**2**) used a similar method except that the amino acids were first separated into acidic, basic, and neutral amino acids on ion exchange resins before chromatography. In the case of 2-directional chromatograms, Block determined the quantity of amino acid by multiplying the maximum color density by the area of the spot.

BLOCK (**3**) has developed this method further in order to obtain the percentage composition of an amino acid mixture. The method is more adapted to relative rather than absolute values. The maximum intensity of a given spot was found to give an estimate of the quantity of each amino acid. The maximum color density of the spot obtained for each amino acid was determined at the same molar concentration. The ratio of the color density for a particular amino acid to the sum of the color densities of all the amino acids constitutes a "standard color ratio for the amino acid in question." An "experimental color ratio" for each amino acid is determined in the same way on a sample of the mixture to be analyzed. The "experimental color

ratio" divided by the "standard color ratio" gives the molar ratio of each amino acid in the mixture. Analyses of casein by this empirical method agreed closely with published values, although a large number of chromatograms (**25**) were necessary to achieve this. (For later work see BLOCK, **3a**.)

ROCKLAND and DUNN (**20**) have used a densitometric method for glycine and alanine. Using 1-directional chromatograms, the amino acids were separated and the color produced by ninhydrin. By measuring the density of an area larger than the biggest spot it was possible to measure the density due to the amino acid by difference.

In the analysis of amino acids in urine by chromatography, BERRY and CAIN (**1a**) utilized visual comparisons of spots. Owing, however, to the effect on the chromatography of water-soluble materials in the urine, the standards with which comparisons were to be made were prepared by adding known amounts of amino acid to urine. Although the authors claim 10 to 15% accuracy for their methods the recovery of added amino acid varied over as wide a range as 63 to 160%.

A novel method somewhat analogous to that of BULL *et al.* (**4**) was introduced by KESTON *et al.* (**12**). The amino acids were made to react with a compound containing radioactive iodine and the resulting compounds were chromatographed in *n*-pentanol saturated with 2 *N* ammonium hydroxide. The radioactivity was measured in the direction of solvent movement, and specific compounds were located and estimated by the local intensity of radioactivity. The methods cited to this point have, in general, estimated the amino acids directly on the paper. These methods have the disadvantage that most utilize only 1-directional separation of amino acid which is often inadequate. If 2-directional chromatography is used, large numbers of chromatograms must be made in order to obtain satisfactory data. This is probably due to insufficient control of the variables involved in the chromatography. Other methods locate amino acids on the paper, then remove the amino acid from the paper and complete the quantitative estimation *in vitro*.

MARTIN and MITTELMAN (**14**) rejected both Folin's color reaction and the ninhydrin reaction for the quantitative determination of amino acids after separation by paper partition chromatography. Microkjeldahl procedure and titration of the amino acids in glacial acetic acid were also tried and found wanting. They finally dissolved the amino acids from the paper, mixed them with insoluble copper phosphate and determined, polarographically, the copper which dissolved by forming a complex with the amino acids. WOIWOD (**29**) used this same general procedure except that he measured the copper colorimetrically. In both of these procedures, 1-directional chromatograms only were used and the amino acids must be located by simultaneous chromatography of known amino acids. WOIWOD (**30**) amplified his earlier work to give a more detailed procedure and has also extended it to include 2-directional chromatography. Woiwod used the copper phosphate procedure to test the ninhydrin reaction, but came to some

misleading conclusions by the use of too dilute a solution of ninhydrin and by failing to recognize that the reaction does not proceed once the paper is dry (*vide infra*).

NAFTALIN (**16**) and AWAPARA (**1**) located the amino acids on the paper by reaction with ninhydrin and then completed the color development in a test tube. The Naftalin procedure is, in our experience, unnecessarily complicated without producing either maximal or reproducible amounts of color. The method of Awapara, particularly as modified by LANDUA and AWAPARA (**13**) is better. This method consists of the following: amino acids are located on the paper with ninhydrin, the amino acid spot is cut out and the reaction is completed by heating in a test tube with MOORE and STEIN'S (**15**) ninhydrin solution which contains stannous chloride. Although this method gives high color production, it has been utilized only with unidirectional chromatograms and gives erratic results, possibly because this ninhydrin solution reacts with ammonia. FOWDEN and PENNEY (**10**) have utilized an analogous procedure except that the amino acids were located by their fluorescence in ultraviolet light after heating the paper to 90 to 100° C. They found that there were losses of amino acids unless the solvents were removed by washing with ether before this treatment.

Variables in the reaction of ninhydrin with amino acids on paper

In the work to devise a quantitative method the ninhydrin reaction has been carried out on the paper. Eventually a method was devised which would, with one spraying and subsequent heating of the paper under controlled conditions, give reproducible and in many cases maximal color. This procedure is simpler and for our purposes has advantages over the Awapara method.

In 1948, the authors (**28**) reported a quantitative method utilizing repeated sprayings (four) with a ninhydrin solution (0.5% in *n*-butanol saturated with water) with 30 minutes heating at 60° C in air between each spraying. Although this method was useful, there were unexplained variations and the need for multiple spraying was then obscure. It is interesting that ROCKLAND and DUNN (**20**) have recently advocated multiple spraying, using up to eight sprays.

It is important to recognize that alanine and serine react with ninhydrin under somewhat different conditions. Most of the other naturally occurring amino acids react either like alanine or serine. Ideally, the reaction conditions should be so chosen that all the amino acids react well. A first thought was that, in this method, the temperature during the heating was a more critical variable than had been previously recognized. That this may have been so is indicated by the data in table I. In table I and subsequent tables and figures where relative color densities are recorded, these are calculated from the readings on the Beckman spectrophotometer. However, it is more convenient and intelligible to have a universal standard of comparison. The one adopted is the color developed from 40 μg. of alanine under the specified

TABLE I

THE NINHYDRIN REACTION ON PAPER: THE EFFECT OF TEMPERATURE ON THE COLOR DEVELOPED BY VARIOUS AMINO ACIDS*

	Relative color density			
	Temperature of air-dry atmosphere			Temperature of alcohol saturated atmosphere
Amino acid	40° C	60° C	80° C	60° C
Alanine	1.000	.942	.737	.696
Serine	1.000	.811	.508	1.061
Threonine	1.000	1.000	.695	....
Glycine	1.000	1.046	....	....

* Forty micrograms of each amino acid were used. The paper was sprayed four times with 0.5% ninhydrin in *n*-butanol saturated with water. After each spraying, the paper was heated in either dry air or alcohol-saturated air at the stated temperature. Color was dissolved in 10 ml. of 50% alcohol and read at 570 mμ with 1 cm. path length.

conditions when the color is dissolved in 10 ml. of solvent and read at 570 mμ at a band width of 1 mμ and a path length of 1.00 cm. This amount of color is arbitrarily set at 1.00 and others expressed relative to it.

The results in table I show that temperature has a marked effect on the color produced in contact with air. However, it was surprising that the color decreased as temperature increased under air-dry conditions since increasing temperatures usually increase the rate of reactions. This suggested that at the higher temperatures the paper dried so rapidly that the solvent disappeared before the color reaction had gone to completion and that this accounted for the apparent benefit of repeated sprayings. To check this idea, the color was formed by heating in an alcohol-saturated atmosphere (see right-hand column in table I). The results show that the problem is not simple since the serine values were higher under saturated conditions while the alanine values were lower.[2]

However, the need for multiple spraying of ninhydrin was apparently to prolong the period, during heating, when the paper was moist. Consequently, amino acid standards were sprayed once with a 2% ninhydrin solution and subsequently heated and the rate of drying was varied by changing the vapor pressure of alcohol in the atmosphere. Figure 1 presents the results which show that the rate of drying does markedly affect color production. Figure 1 also shows that there is no way of producing maximal color simultaneously with both alanine and serine by controlling the rate of drying. It also indicates that the color produced may vary considerably depending on the drying conditions in the oven.

[2] This fact stresses that all amino acids do not react alike to ninhydrin. According to the theory of RUHEMANN (19), all amino acids should react alike to give the same compound and the same amount of color per atom of amino nitrogen. MOORE and STEIN (15) have shown that this is not so even under their carefully specified conditions. The differences between amino acids are even more accentuated when the ninhydrin reaction is carried out on a filter paper sheet (see table IX).

Alanine gave more color under rapid than under slow drying conditions (fig. 1). Therefore, a short period, when the paper was wet, sufficed for the reaction and, once the paper was dry, subsequent decomposition was reduced. If the paper stayed wet, under slow drying conditions, the colored compound decomposed after it had been formed. Serine, on the other hand, produced more color under conditions where the rate of drying was intermediate. Hence, serine reacts more slowly than alanine and a reduced rate

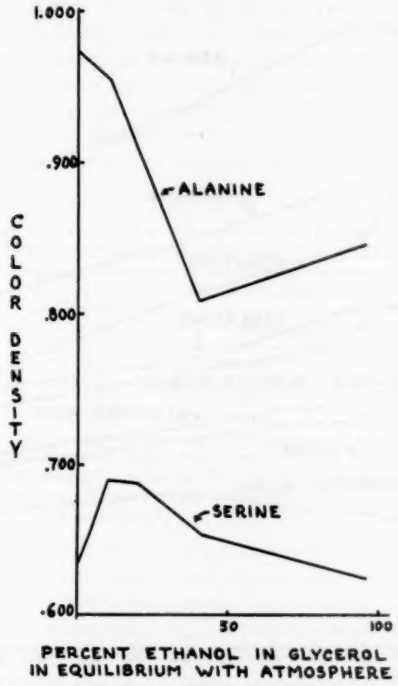


FIG. 1. Ninhydrin reaction on paper: effect of rate of drying or vapor pressure of alcohol on the color produced by amino acids and ninhydrin.

of drying results in more color; the increased production of color more than offsets its destruction.

Figure 2 shows that the alanine-ninhydrin color (as well as that from other acids) does decompose rapidly under moist air conditions. Figure 2 also shows that the rate of destruction of the serine color is not greater than its rate of formation until after 20 minutes: whereas, for alanine, this occurs before five minutes. This is consistent with differences between alanine and serine (see table I) previously found.

The first thought was that decomposition of the ninhydrin-amino acid color was caused by oxygen. If this were so, then it should be possible to produce conditions under which both alanine and serine could be made to give maximum color. That the destruction of the ninhydrin color is due to oxygen is shown in table II. The slightly reduced color under nitrogen was probably due to the fact that a small fraction of oxygen remained. It is clear that, in the presence of oxygen, the ninhydrin color formed with both

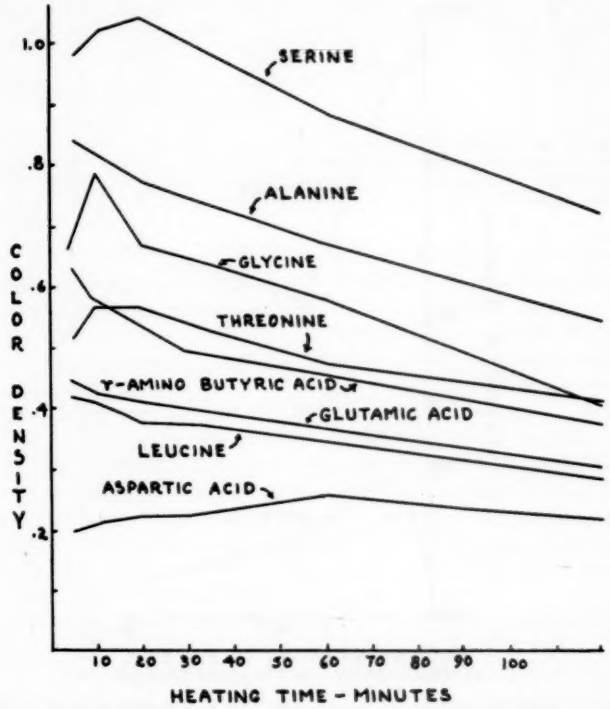


FIG. 2. Ninhydrin reaction on paper: effect of time on the color developed between ninhydrin and amino acid under aerobic conditions in an alcohol-saturated atmosphere at 60° C.

alanine and serine is destroyed but the serine color to a lesser extent. The effect of dissolved oxygen on the ninhydrin reaction in test tubes was noted independently by MOORE and STEIN (**15**), and this led them to incorporate a reducing agent in the reaction mixture.

It seemed that the lower rate of destruction of the serine-ninhydrin color might be due to the non-volatile aldehyde formed in the reactions, after RUHEMANN (**19**). Alanine should form a volatile aldehyde which would not

protect the color against decomposition. However, the addition of a non-volatile aldehyde (phenylacetaldehyde) did not reduce the rate of destruction of the alanine color.

Therefore, the best conditions for the ninhydrin reaction to occur on the paper are as follows:

(I) The paper should be heated anaerobically.
(II) The paper should be heated in an alcohol-saturated atmosphere.

To achieve these ends, a special apparatus was built (see fig. 3) to accommodate full sized ($18\frac{1}{2}'' \times 22''$) 2-dimensional chromatograms so that they may be heated under alcohol-saturated, anaerobic conditions. The apparatus was so designed that a heavy gas (carbon dioxide) would flow upwards on both sides of the chromatogram. This prevents oxygen from diffusing down into the chamber where the filter paper is heated. This apparatus consisted principally of an outer cabinet and an inner chamber.

TABLE II

NINHYDRIN REACTION ON PAPER: THE EFFECT OF AEROBIC AND ANAEROBIC CONDITIONS ON THE DEVELOPMENT OF COLOR.*

Amino acid	Relative color density		
	Atmosphere		
	Air	Nitrogen	Carbon dioxide
Alanine	.751	.980	1.000
Serine	.857	.967	1.000

*Forty micrograms of amino acids were used. Papers were sprayed once with 2% ninhydrin in 95% ethanol containing 2% collidine-lutidine and heated for 30 minutes at 60° C under stated conditions. Readings were made on 10 ml. of solution at 570 mμ with 1 cm. path length.

The inner chamber is kept saturated and anaerobic for actual development of the color, while the outer cabinet maintains a constant temperature in the inner chamber. The outer cabinet has a circulating fan (D), a space heater (E), and a temperature regulator (C). The inner chamber is inserted in the top of the cabinet and has its own lid. Along the bottom of the inner chamber is a perforated pipe (F) to provide an even flow of carbon dioxide. The pipe is immersed in alcohol and a shield (G) prevents splashing the chromatographed papers. At the top of the inner chamber are two shallow troughs (H) on the outside of which hang filter papers (I), which dip into alcohol at the bottom: alcohol is also placed in the upper troughs and travels down, as well as up, these papers to keep the atmosphere saturated. The alcohol is placed in the upper troughs (H) and allowed to permeate the saturating papers (J) before the ninhydrin-treated paper is inserted. No free alcohol remains in the trough (H) so that there is no continuous flow over the saturating paper during development of the color.

After being sprayed with ninhydrin, the chromatographed paper is placed in the inner chamber so that it extends well down and is kept wet and anaer-

obic at a constant temperature. Under these conditions, the amino acids form a color quickly, the maximum intensity occurring in most cases before 30 minutes (see fig. 4). Except in the case of glycine and aspartic acid, most of the color has formed in five minutes. However, in order to be sure that the reaction is completed, a 20- or 30-minute heating period at 60° has normally been used.

Figure 4 suggests that temperatures higher than 60° C, within the limits set by the boiling point of ethanol, would be of no avail since the reaction

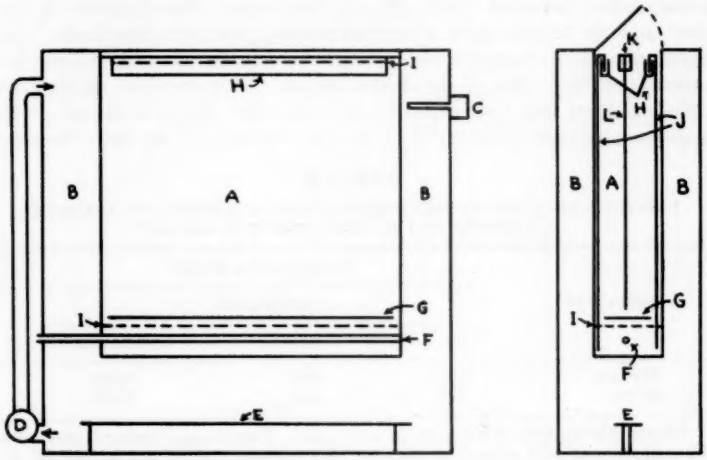


FIG. 3. Color development cabinet. A. Inner closed chamber for control of saturated atmosphere. B. Outer cabinet for temperature control. C. Thermoregulator. D. Circulating fan—arrows indicate direction of air flow. E. Space heater. F. Inlet for carbon dioxide to inner chamber—carbon dioxide bubbles through alcohol. G. Baffle plate to prevent splashing of alcohol onto papers. H. Trough to hold filter paper and alcohol in order to maintain alcohol-saturated conditions at the top of the inner chamber. I. Alcohol level. J. Extra filter papers, wet with alcohol, for saturating the inner chamber. K. Clamp to hold filter paper chromatogram. L. Filter paper chromatogram.

is rapidly completed. Enough tests have been made at temperatures as high as 70° C and below 60° C to show that the main effect of temperature is on the rate of the reaction—not on the final amount of color developed.

Since the destruction of the color, when the paper is wet, is rapid under aerobic conditions, the question then is whether the filter paper sheet should also be dried under anaerobic conditions. Table III shows that there is very little difference in the color whether the paper is dried in air or carbon dioxide. In fact, the close agreement between these two sets of figures strongly indicates that the variables involved in the ninhydrin reaction on paper have now been brought under control.

Once the paper is dry, the subsequent rate of destruction of the ninhydrin color, in air at room temperature, is very low, as shown in table IV. Over a period of several days, the loss in color is negligible, which is in sharp contrast to the rapid destruction under moist conditions in air. This result allows the final readings to be deferred up to two or three days, if

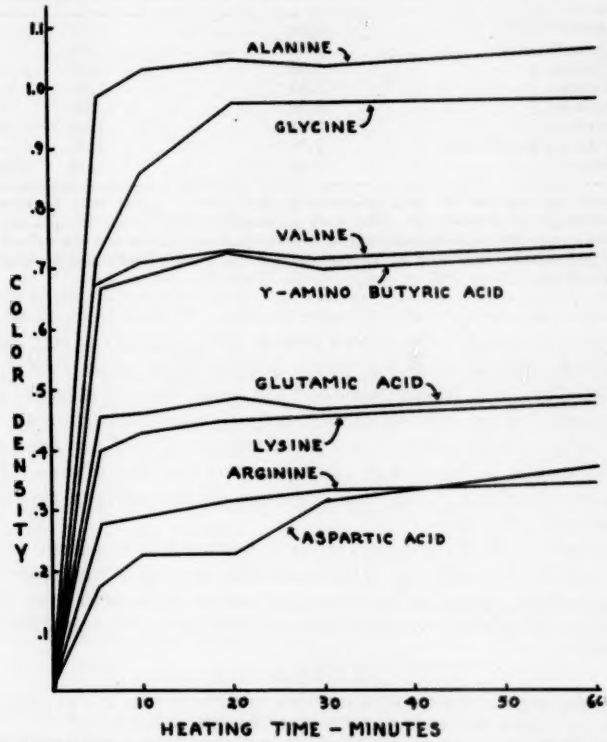


FIG. 4. Ninhydrin reaction on paper: effect of time of heating on color development between amino acids and ninhydrin. Forty micrograms of amino acid were sprayed once with 3% ninhydrin and heated at 60° C in CO_2 atmosphere.

necessary, without loss of color. However, since the background color increases considerably (2 to 3 times) in 24 hours, the possibility of greater blank error makes it undesirable to defer readings for more than a few hours.

After extraction from the paper the color does not fade rapidly in solution and is more stable than that produced by the Moore and Stein procedure (table V). In practice, the solvent (50% ethanol with water) is added

TABLE III

THE NINHYDRIN REACTION ON PAPER: THE EFFECT OF AEROBIC AND ANAEROBIC DRYING FOLLOWING DEVELOPMENT OF COLOR ON THE PAPER*

Amino acid	Color density	
	Dried in air	Dried in carbon dioxide
Aspartic acid	1.09	1.00
Glutamic acid	1.02	1.00
Glycine	1.03	1.00
Alanine	1.03	1.00
Arginine	0.96	1.00
Lysine	0.97	1.00
γ-Amino butyric acid	1.00	1.00
Valine	1.03	1.00

*Forty micrograms of each amino acid were used. Color was developed in alcohol-saturated atmosphere under carbon dioxide at 60° C for 30 minutes after one spray with 2% ninhydrin dissolved in 95% alcohol. Color was dissolved in 10 ml. of 50% alcohol and read at 570 mμ with 1 cm. path length. Each value is an average of two.

just before making spectrophotometer readings. Within 15 to 30 minutes, the color is dissolved. The colored product is very soluble in 50% ethanol with water, although much less soluble in either water or absolute alcohol. The solubility of the colored compound will never limit the accuracy of the determination under ordinary chromatographic conditions. Ten milliliters of solvent is usually adequate and is convenient. For many weak spots, the color may be dissolved in 20 ml. volume and read in a colorimeter tube with a path length of 10 cm., thus increasing the sensitivity of the reading approximately five times.

CONSDEN *et al.* (**5**) originally utilized *n*-butanol as a solvent for ninhydrin and subsequently (**6**) pointed out that more color was produced if the butanol is saturated with water: our results agree with this. While some effect of the pH of the water with which the butanol is mixed has been

TABLE IV

THE NINHYDRIN REACTION ON PAPER: THE EFFECT OF TIME ON THE LOSS OF COLOR ON PAPER AT ROOM TEMPERATURE IN THE DARK*

Time—hours	Relative color density
0	1.000
24	1.000
64	.994
118	.992
237	.893
840	.768

*Forty micrograms of alanine were used. Color was developed by spraying with a 2% solution of ninhydrin in 95% alcohol with 2% collidine-lutidine 1 : 3 and heated at 60° C for one half hour in an alcohol-saturated anaerobic atmosphere. Color was dissolved at intervals in 10 ml. of 50% alcohol and read at 570 mμ with 1.0 cm. path length.

TABLE V

NINHYDRIN REACTION ON PAPER: THE EFFECT OF TIME ON THE NINHYDRIN COLOR IN A SOLUTION OF 50% ALCOHOL*

Amino acid	Percentage decrease in color intensity in dark in 15 hrs. at 25° C	
	Color developed by method of Moore and Stein (15)	Color developed on paper
Threonine	12.2	3.40
Alanine	12.8	1.68
Leucine	15.8	2.67
Aspartic acid	12.3	4.63
Serine	14.2	0.99
Glutamine	11.3	1.54
γ-Amino butyric acid	10.5	1.94
Valine	10.8	1.21

*Forty micrograms of amino acids were used. Final volume was 10 ml. in both procedures and readings were made at 570 mμ with a 1 cm. path length. Color was developed on paper by one spray with 2% ninhydrin in 95% alcohol containing 2% collidine-lutidine (1:3) and heated one half hour at 60° C in a carbon dioxide atmosphere.

observed, it has been found that if the amino acid has been previously treated with collidine-lutidine, as in chromatography, the pH of the ninhydrin solution has no effect between pH 4.0 and 8.0—though beyond these limits the intensity of color decreases markedly. For certain unchromatographed amino acids (*e.g.* glutamic acid) collidine-lutidine applied in the alcohol-ninhydrin solution gave as good results as pre-treating them with collidine-lutidine. However, color production from other amino acids is benefited (table VI) by the collidine-lutidine in the ninhydrin solvent. Hence,

TABLE VI

NINHYDRIN REACTION ON PAPER: THE EFFECT OF COLLIDINE-LUTIDINE IN THE NINHYDRIN SOLVENT ON THE COLOR PRODUCED WITH VARIOUS AMINO ACIDS*

Amino acid	Relative color density		
	No collidine-lutidine (1:3)	Collidine-lutidine (1:3) applied to amino acid spot prior to application of ninhydrin solution	Collidine-lutidine (1:3) applied to amino acid spot in ninhydrin solution
Glutamic acid	.948	.992	1.000
Threonine	.980	.960	1.000
Alanine	.903	.988	1.000
Leucine	.869	.898	1.000
Aspartic acid	.877	.888	1.000
γ-Amino butyric acid	.828	.987	1.000

*Color was developed by spraying amino acids (40 μg.) on paper with a 2% solution of ninhydrin in 95% alcohol, and heating at 60° C for 30 minutes in a carbon dioxide atmosphere. Color was dissolved in 50% ethanol and read at 570 mμ with 1 cm. path length.

collidine-lutidine (1:3) is added to the alcohol solution of ninhydrin and presumably its effect is due to a buffering action.

Ethyl alcohol was found to be a satisfactory solvent for ninhydrin (see table VII). Seventy-five per cent. ethanol not only gives less color, but also has the disadvantage that it is such a good solvent for the colored product that spots run together during development. Since 95% alcohol gave as much color as *n*-butanol saturated with water, the former is now used as the solvent for ninhydrin.

TABLE VII

THE NINHYDRIN REACTION ON PAPER: THE EFFECT OF NINHYDRIN SOLVENT ON COLOR PRODUCED*

Amino acid	Relative color density		
	Solvent for ninhydrin		
	75% ethanol with 2% collidine-lutidine	95% ethanol with 2% collidine-lutidine	*n*-Butanol saturated with water and containing 2% collidine-lutidine
Glutamic acid	.913	1.000	.991
Serine	.980	1.000	1.023
Alanine	.974	1.000	.982
γ-Amino butyric acid	.946	1.000	1.000

*Color developed by spraying amino acid (40 μg.) on paper with 2% ninhydrin solution and paper was heated for 30 minutes at 60° C in a carbon dioxide atmosphere saturated with alcohol. Colors read in 10 ml. of solution at 570 mμ with 1 cm. path length.

The proper concentration for the ninhydrin cannot be stated categorically. However, if there is less than 4 μg. of amino nitrogen per sq. cm. of Whatman no. 1 filter paper, a complete reaction will be obtained with 1% ninhydrin solution (fig. 5). One would never have a higher concentration than this because chromatographic separation on the paper would then be inadequate. Woiwod (**30**), on the other hand, has suggested that in the reaction of ninhydrin with glycine, the color formation is inhibited by some product of the reaction. Knowing the high concentration of ninhydrin needed for complete reaction, it is not surprising that Woiwod did not achieve this.

The absorption spectra of the reaction products

The colored product from the usual reaction of ninhydrin with an amino acid has two absorption peaks; one at 410 mμ and one at 570 mμ. Although the peak at 410 mμ is higher, the higher blank color at this wave length renders it less suitable (see fig. 6). Consequently, readings have been made at 570 mμ with 1-cm. path lengths. At this wave length, the color follows Beer's law accurately (table VIII).

Since all the ninhydrin spots after chromatography are not the same

size, it is essential to make a different blank correction in each determination. Once the amino acid color has been developed on the paper, the spot is cut out and weighed to the nearest milligram on a Roller-Smith balance. Areas with no spots are also cut out and weighed to determine the blank correction. The blank color per unit weight of paper is determined. Since the weight per unit area of the paper is uniform, it only requires that the

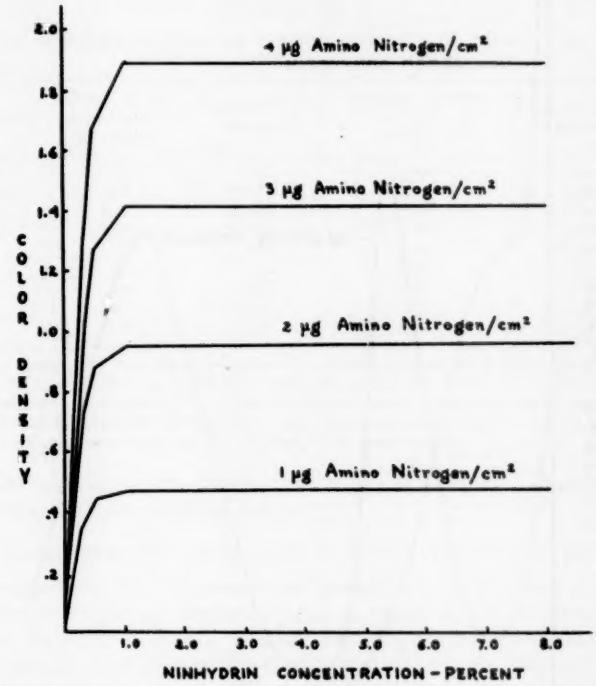


FIG. 5. Ninhydrin reaction on paper: effect of concentration on color developed by various concentrations of amino nitrogen. Alanine was used as source of amino nitrogen. Tests were done for 30 minutes at 60° C.

blank color follows Beer's law and that the spraying be uniform so that the density corrections are proportional to the amount of paper. It has been found that the blank color does in fact follow Beer's law at 570 mμ and this blank correction is, therefore, valid.

The above procedure is applicable to all those amino acids which give a characteristic purple test with ninhydrin (*i.e.*, glutamic acid, serine, threonine, alanine, glutamine, lysine, arginine, γ-amino butyric acid, valine, leucine). Certain amino acids which give a blue color on the paper still

show almost exactly the same absorption bands when the color is extracted (*i.e.*, aspartic acid, β-alanine): this is also true of others which, under certain conditions, react more brown (tyrosine, glycine, and phenyl-alanine).

It is known that hydroxyproline and proline do not react with ninhydrin to form the same compound as the above amino acids (**11, 14, 18**). Aspara-

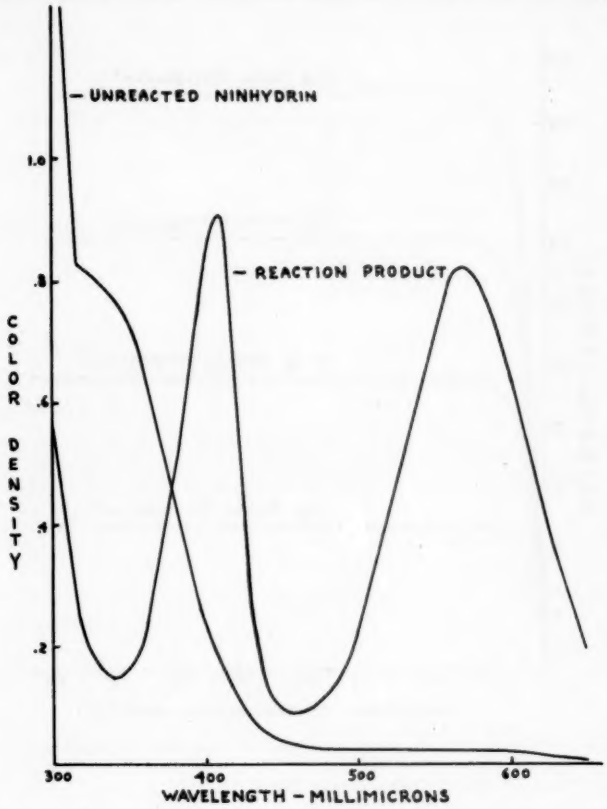


FIG. 6. Ninhydrin reaction on paper: absorption spectra of the product of the reaction of ninhydrin with amino acid and unreacted ninhydrin.

gine also reacts with ninhydrin on paper to give a color similar to that of proline and hydroxyproline (fig. 7). A comparison of figure 7 with the curve published by MOORE and STEIN (**15**) indicated that the reaction may not be the same on paper as in the test tube. When the ninhydrin reaction is performed on the paper, the proline product has peaks at 330 and 410 mμ, the

hydroxyproline product at 330 mμ, and the asparagine product at 317 and 350 mμ. Even though the blank color is relatively high in this region (see fig. 6), proline, hydroxyproline, and asparagine colors from the ninhydrin reaction are read at 330 mμ with a band width of .7 to .8 mμ. To obtain accurate readings, the ninhydrin must be sprayed on the paper very evenly since the blank correction is so high. Asparagine is read at 330 mμ rather

TABLE VIII

THE NINHYDRIN REACTION ON PAPER: THE EFFECT OF QUANTITY OF AMINO ACID ON THE COLOR PRODUCED*

Amino acid	Quantity of amino acid-micro gm.	Observed color density	Relative amount of color obtained	Relative amount of amino acid
Alanine	40	.892	1.00	1.00
Alanine	80	1.773	1.99	2.00
Alanine	120	2.668	2.99	3.00
Alanine	160	3.556	3.98	4.00
Serine	20	.384	1.00	1.00
Serine	40	.765	1.99	2.00
Serine	60	1.165	3.03	3.00
Serine	80	1.536	4.00	4.00
Serine	120	2.284	5.94	6.00

*The color was developed on paper by spraying amino acid spots once with a 2% solution of ninhydrin in 95% alcohol containing 2% collidine-lutidine (1:3). The paper was heated at 60° C in an alcohol-saturated atmosphere made anaerobic with carbon dioxide for 30 minutes. The color was dissolved in 10 ml. of 50% ethanol and readings made at 570 mμ with 1 cm. path length.

than 350 mμ because it is convenient to read it at the same wave length as the proline and hydroxyproline colors.

A quantitative procedure for the ninhydrin reaction on paper

The procedure for the quantitative formation and measurement of the color formed by nitrogen compounds and ninhydrin on paper is as follows:

The ninhydrin solution is sprayed evenly on the paper chromatogram in the form of a 1 or 2% solution in 95% ethanol containing 2% of a collidine-lutidine mixture (1:3). The spraying is conveniently performed with a small hand paint sprayer activated by compressed air. A sprayer delivering about 45 ml./min. is satisfactory and about 55 ml. are sprayed upon a sheet of Whatman no. 1 filter paper 18 by 22 inches. Any excess of solvent which causes the paper to "shine" may cause the spots to spread diffusely during the heating. Therefore, before the paper chromatogram is placed in the color development chamber (see fig. 3) any excess of solvent causing a "shine" is allowed to evaporate. The papers are hung vertically in a chamber, which exposes them for 30 minutes to an anaerobic (CO_2) atmosphere in equilibrium with free liquid ethanol at 60° C, and in such a way that the

initial spot and most of the amino acids are on the lower half of the chromatogram. (Before use, the chamber is tested with a glowing splinter to show that it is anaerobic.)

After color development, the paper is removed from the chamber and dried at room temperature in air. The ninhydrin-colored areas are cut out, weighed to one milligram, cut into pieces about $\frac{1}{4}$ by $\frac{3}{4}$ inch, and extracted

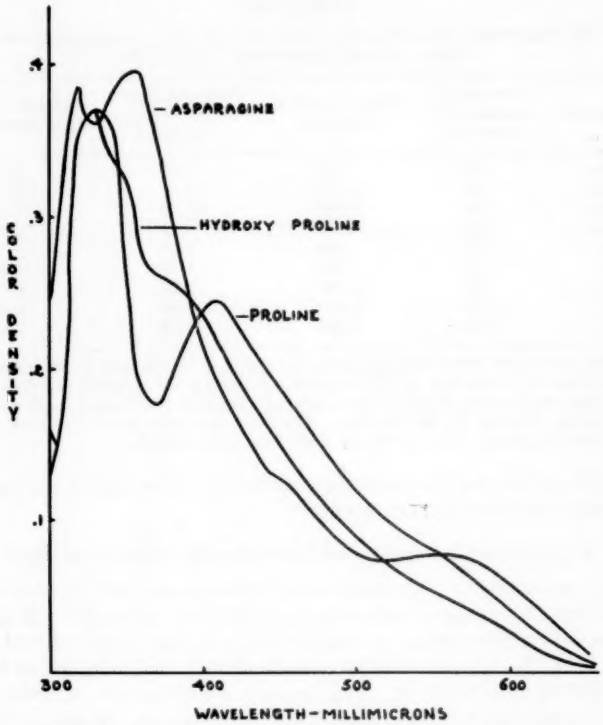


FIG. 7. Ninhydrin reaction on paper: absorption spectra of the product of the reaction of ninhydrin with proline (80 μg.), hydroxyproline (80 μg.), and asparagine (120 μg.).

in test tubes ($\frac{3}{4}$ inch) with 10 ml. of 1:1 ethanol-water mixture. In the extraction, only gentle agitation (*i.e.*, several inversions of the test tube) is practiced because lint comes off the paper with shaking, and the turbidity affects the density readings. In all these operations, fastidious care is necessary to avoid contaminating the paper from the hands or by dust. The solution of the colored compound is complete in 30 minutes at room temperature. The color may be read at any time thereafter up to several hours, since fading is slow (see table V). The color density is measured in a Beckman spectrophotometer with 1-cm. path length at 570 mμ (band width, 1 mμ)

for blue and purple spots and at 330 mμ (band width, .7–.8 mμ) for orange-brown spots. A blank determinition is made on an uncolored area of the same paper and a blank correction for each spot is calculated from the weight of paper. The corrected reading is proportional to the amount of the individual amino acid.

Tests of the quantitative procedure

Table VIII shows that the amount of color produced under the conditions described above is almost exactly proportional to the amount of amino acid used.

A further test was to compare the color produced by the above procedure using a number of amino acids with that obtained by Moore and Stein's reaction method (see table IX).

These results indicate that the paper method gives comparable amounts of color to that obtained in the test tube by Moore and Stein's method. The most notable exception is tyrosine. The behavior of asparagine on the paper

TABLE IX

THE NINHYDRIN REACTION ON PAPER: A COMPARISON OF COLOR PRODUCED ON THE PAPER AND IN THE TEST TUBE BY THE METHOD OF MOORE AND STEIN (15)*

Amino acid	Relative color developed per mole in test tube	Relative color developed per mole on paper	Color developed on paper as a % of color developed in test tube
Leucine	1.00	.96	96
Cysteic acid	1.00	.86	86
Aspartic acid	.88	.75	86
Glutamic acid	1.05	.98	93
Serine	.94	.94	100
Glycine	1.01	.88	88
Threonine	.92	.88	95
Alanine	1.01	.99	98
Histidine	1.04	.80	77
Lysine	1.12	.97	87
Arginine	1.00	.89	89
Methionine sulphone	1.00	.78	78
Methionine sulphoxide	1.00	.72	72
Valine	1.02	1.03	101
Tryptophan	.72	.66	92
Tyrosine	.88	.44	50
γ-Amino butyric acid	1.00	.89	89

*Color developed in test tube using 40 μg. of amino acid, according to Moore and Stein's procedure (15). The volume was made to 10 ml. with 50% alcohol, and the densities per mole expressed relatively to leucine. Color on paper was developed by spraying once with 2% ninhydrin in 95% alcohol containing 2% collidine-lutidine. Paper was heated for 30 minutes at 60° C in an anaerobic (CO_2) ethanol saturated atmosphere. The color was extracted with 10 ml. of 50% ethanol and readings made at 570 mμ with 1 cm. path length, and the color density expressed relative to the density of an equimolar leucine solution treated in the test tube by the Stein and Moore method.

is anomalous since it gives a brown color with ninhydrin. However, the most important point is that the values obtained by the paper method are closely reproducible (see table X).

The following amino acids, all of which occur free in plants, react reproducibly with ninhydrin on paper under the conditions prescribed and form blue colors determinable at 570 mμ: cysteic (after oxidation of cystine by hydrogen peroxide), aspartic, glutamic and γ-amino butyric acids; glycine, serine, threonine, alanine, glutamine, lysine, arginine, methionine sulphoxide and sulphone, valine, the leucines, phenylalanine, tyrosine, and tryptophan. Asparagine, proline, and hydroxyproline behave reproducibly but their

TABLE X

THE NINHYDRIN REACTION ON PAPER: THE VARIATION IN COLOR PRODUCED BY VARIOUS AMINO ACIDS WITH NINHYDRIN ON PAPER*

Amino acid	Number of analyses	Standard deviation as per cent. of average colorimeter reading
Glutamic acid	30	2.34
Serine	12	0.97
Glycine	28	2.20
Threonine	22	1.75
Alanine	23	1.68
Leucine	20	2.40

*Color was developed by spraying amino acids (40 μg.) on paper with a 2% solution of ninhydrin in 95% alcohol and heating at 60° C for 30 minutes in a carbon dioxide atmosphere. The color was dissolved in 50% ethanol and read at 570 mμ with 1 cm. path length.

brown colors are determined at 330 mμ where the blank correction is somewhat greater. With these last three substances, the color produced per unit weight of substance is also less.

The work of this and the succeeding papers of the series has been assisted in a variety of ways. For two years one of us (J. F. T.) held a Post-Doctoral Fellowship of the National Institute of Health. Apparatus and special facilities have been made available to one of us (F. C. S.) under specific grants from the National Institute of Health and technical assistance was furnished in part under a grant from the Growth Committee of the National Research Council. The association of Mr. R. M. Zacharius with the work was made possible by a grant to one of us (F. C. S.) from the Nutrition Foundation. The full development of the work, in the form to be described, would not have been possible without the interest of the Beechnut Packing Company in the work of the Botany Department at Rochester during the years in question. For all this help we acknowledge our debt.

Summary

1. The variables involved in the reaction of ninhydrin with amino acids

on paper have been individually investigated and finally controlled. The principal variables are: temperature, humidity, access to oxygen, concentration of ninhydrin and the solvent used to apply it to the paper.

2. The conditions under which the ninhydrin reaction may be carried out quantitatively on paper have been established and described and an apparatus for the purpose has been designed.

3. The method prescribed has been compared with others which have been suggested.

4. The quantitative procedure as described is now suitable for application to partition chromatography on paper of those amino acids found in plants.

DEPARTMENT OF BOTANY
UNIVERSITY OF ROCHESTER
ROCHESTER, NEW YORK

LITERATURE CITED

1. AWAPARA, J. Application of paper chromatography to the estimation of some free amino acids in tissue of the rat. Jour. Biol. Chem. **178**: 113–116. 1949.

1a. BERRY, H. K. and CAIN, L. Biochemical individuality. IV. A paper chromatographic technique for determining excretion of amino acids in the presence of interfering substances. Arch. Biochem. **34**: 179–189. 1949.

2. BLOCK, R. J. Quantitative estimation of amino acids on paper chromatograms. Science **108**: 608–609. 1948.

3. BLOCK, R. J. Quantitative paper chromatography: a simplified procedure. Proc. Soc. Exptl. Biol. and Med. **72**: 337–341. 1949.

3a. BLOCK, R. J. Estimation of amino acids and amines on paper chromatograms. Anal. Chem. **22**: 1327–1332. 1950.

4. BULL, H. B., HAHN, J. W., and BAPTIST, V. H. Filter paper chromatography. Jour. Amer. Chem. Soc. **71**: 550–553. 1949.

5. CONSDEN, R., GORDON, A. H., and MARTIN, A. J. P. Qualitative analysis of proteins: a partition chromatographic method using paper. Biochem. Jour. **38**: 224–232. 1944.

6. CONSDEN, R., GORDON, A. H., and MARTIN, A. J. P. The identification of lower peptides in complex mixtures. Biochem. Jour. **41**: 590–596. 1947.

7. DENT, C. A. The amino-aciduria in Fanconi Syndrome. A study making extensive use of techniques based on paper partition chromatography. Biochem. Jour. **41**: 240–253. 1947.

8. DENT, C. E., STEPKA, W., and STEWARD, F. C. Detection of free amino-acids of plant cells by partition chromatography. Nature **160**: 682–683. 1947.

9. FISHER, R. B., PARSONS, D. S., and MORRISON, G. A. Quantitative paper chromatography. Nature **161**: 764–765. 1948.

10. Fowden, L. and Penney, J. R. Elimination of losses in the quantitative estimation of amino-acid by paper chromatography. Nature **165**: 846–847. 1950.
11. Grassman, W. and von Arnim, K. Reaction of ninhydrin and isatin with proline and hydroxyproline. Ann. Chem. **509**: 288–303. 1934.
12. Keston, A. S., Udenfriend, S., and Levy, M. Paper chromatography applied to the isotopic derivative method of analysis. Jour. Amer. Chem. Soc. **69**: 3151. 1947.
13. Landua, A. J. and Awapara, J. Use of modified ninhydrin reagent in quantitative determination of amino acids by paper chromatography. Science **109**: 385. 1949.
14. Martin, A. J. P. and Mittelman, R. Quantitative micro-analysis of amino-acid mixtures on paper partition chromatograms. Biochem. Jour. **43**: 353–358. 1948.
15. Moore, S. and Stein, W. H. Photometric ninhydrin method for use in chromatography of amino-acids. Jour. Biol. Chem. **176**: 367. 1949.
16. Naftalin, L. Quantitative chromatographic estimation of α-amino-acids. Nature **161**: 763. 1948.
17. Polson, A. Quantitative partition chromatography and the composition of *E. coli*. Biochim. et Biophys. Acta **2**: 575–581. 1948.
18. Polson, A., Moseley, V. M., and Wyckoff, R. W. G. The quantitative chromatography of silk hydrolysate. Science **105**: 603–604. 1947.
19. Ruhemann, S. Triketohydrindene hydrate. III. Its relation to alloxan. Jour. Chem. Soc. **99**: 792–800. 1911.
20. Rockland, L. B., and Dunn, M. S. Quantitative determination of amino acids on filter paper chromatograms by direct photometry. Jour. Amer. Chem. Soc. **71**: 4121–4122. 1949.
21. Steward, F. C., Berry, W. E., Preston, C., and Ramamurti, T. K. The absorption and accumulation of solutes by living plant cells. X. Time and temperature effects on salt uptake by potato discs and the influence of storage conditions of the tubers on metabolism and other properties. Annals of Botany N.S. **7**: 221–260. 1945.
22. Steward, F. C. and Preston, C. Metabolic processes of potato discs under conditions conducive to salt accumulation. Plant Physiol. **15**: 23–61. 1940.
23. Steward, F. C. and Preston, C. The effect of salt concentration upon the metabolism of potato discs and the contrasted effect of potassium and calcium salts which have a common ion. Plant Physiol. **16**: 85–116. 1941.
24. Steward, F. C., Stout, P. R., and Preston, C. The balance sheet of metabolites for potato discs showing the effect of salts and dissolved oxygen on metabolism at 23° C. Plant Physiol. **15**: 409–447. 1940.

25. STEWARD, F. C. and STREET, H. E. The soluble nitrogen fractions of potato tubers; the amides. Plant Physiol. **21**: 155–193. 1946.
26. STEWARD, F. C. and STREET, H. E. The nitrogenous constituents of plants. Ann. Rev. Biochem. **16**: 471–502. 1947.
27. STEWARD, F. C. and THOMPSON, J. F. The nitrogenous constituents of plants with special reference to chromatographic methods. Ann. Rev. Plant Physiol. **?**: 233–264. 1950.
28. THOMPSON, J. F. and STEWARD, F. C. A quantitative procedure for the determination of amino acids in plants using partition chromatography on paper. 23rd Ann. Meeting Amer. Soc. Plant Physiol. Sept. 8–10. 1948.
29. WOIWOD, A. J. Micro estimation of amino-nitrogen and its application to paper partition chromatography. Nature **161**: 169. 1948.
30. WOIWOD, A. J. A method for the estimation of micro amounts of amino nitrogen and its application to paper partition chromatography. Biochem. Jour. **45**: 412–417. 1949.

A BIOCHEMICAL BASIS FOR COPPER–NITROGEN BALANCE IN TUNG

SEYMOUR G. GILBERT

(WITH FOUR FIGURES)

Received June 12, 1950

Introduction

Work on tung (**5, 6**) has clearly established that a close relationship exists between the severity of copper deficiency and the amount of nitrogen fertilizer supplied to the plant. Normal plants free from the symptoms of copper deficiency can be produced under conditions of low nitrogen supply when the copper supply is sufficiently high (fig. 1). When high rates of nitrogen fertilizers are supplied to plants on low copper levels, the plants usually show severe symptoms of copper deficiency, such as necrosis of the leaves and eventual dieback of the terminals (fig. 2). Such affected plants can be made to resume normal growth by application of copper sulphate either to the soil or as a spray to the leaves. Physiological studies (**5**) have shown that copper deficiency is characterized in tung by the formation of abnormal amounts of complex nitrogen compounds at the expense of the carbohydrate reserves of the plant. The result is a net increase in protein content of the copper-deficient material over that of the normal on the basis of either per plant part or percentage of dry weight. LUCAS (**10**) has also reported an abnormally high protein percentage in copper-deficient plants in comparison with those of high copper content.

In the previous work with tung (**5**) protein was determined as hot-water-insoluble nitrogen content, and in the work of LUCAS (**10**) as amino-N content. It is thus impossible to determine whether the high "protein" values obtained represent actual quantitative differences or a qualitative difference in protein composition such as would result from a high ratio of basic polyamino to monoamino acids. Studies were therefore made of the amino acid composition of the proteins in normal and in copper-deficient tung leaves.

Methods

The isolation of protein from the tung leaf is complicated by the presence of a tannin which causes protein flocculation upon cell rupture. A procedure has been developed for determining the protein fraction of the leaves despite the tannin interference.

Freshly minced green leaves are disintegrated with distilled water in a Waring Blendor and then strained through muslin. The water-soluble material and flocculated protein pass through the muslin. The fibrous residue is repeatedly extracted by further Blendor treatment until the filtrate is protein-free. Usually four treatments are used for 20-gm. aliquots of leaves.

The combined filtrates are brought to pH 4.0 with acetic acid and centrifuged. The green pellet obtained is dispersed by adding NaOH to pH 9.0; the material centrifuged again; and the supernatant returned to pH 4.0 with acetic acid. The reprecipitated protein is alternately dissolved at pH 9.0 and centrifuged at pH 4.0 until the pH 4.0 supernatant gives only a faint tannin test with ferric chloride. The purified protein is washed by centrifuging with acetone until the acetone washings are colorless. It is then dried at 70° C in vacuum. The process is conducted under quantitative conditions, all fractions being analyzed for nitrogen, dry matter, and chloro-

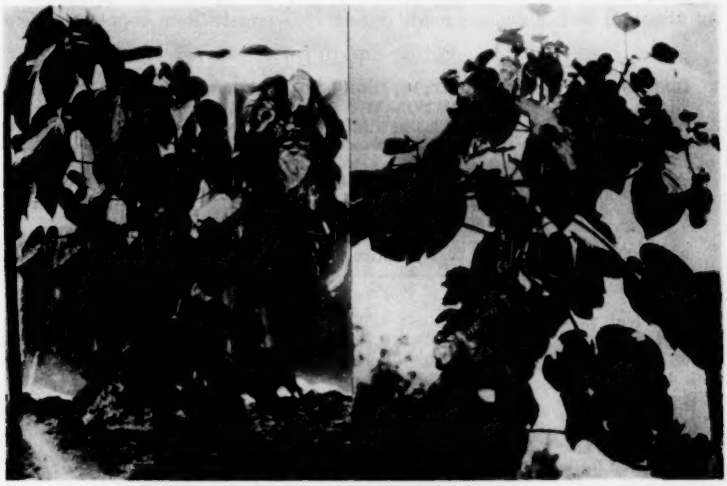


FIG. 1 FIG. 2

FIG. 1. "Normal" tung seedling given high copper, low N treatment.
FIG. 2. "Copper-deficient" tung seedling given low copper, high N treatment.

phyll content. Chlorophyll was determined colorimetrically by using a Cenco photelometer calibrated spectrophotometrically for chlorophyll by a Beckman Model DU spectrophotometer, according to COMAR *et al.* (1).

Results

Data for a representative fractionation of leaf material are given in table I. These data show that about 50 to 60% of the original nitrogen of the leaf was isolated in the form of protein soluble at pH 9.0. The residue from the original four extractions contained about 30% of the chlorophyll of the leaves. From these data and microscopical examination it was evident that the residue contains chromoprotein both in unruptured chloroplasts and adsorbed in the fibers or cellulose, as has also been reported by

CROOK (3). Considerable chlorophyll (30 to 40%) was lost in the process of protein purification. The protein obtained is therefore primarily chromoprotein.

It should be noted that the protein fraction soluble at pH 9.0, as obtained, has only about a 50% purity on the basis of a protein having 16% of N. The associated non-peptide material is probably of carbohydrate derivation and is firmly bound chemically, since hydrolytic reagents are required to remove it.

Considerably more protein was present in the copper-deficient leaves in a form which could be isolated readily. These results are representative of about 20 such isolations made during 1947 and 1948 on copper-deficient

TABLE I

DISTRIBUTION OF DRY WEIGHT, NITROGEN, AND CHLOROPHYLL IN FRACTIONS MADE IN PROTEIN ISOLATION FROM NORMAL AND FROM COPPER-DEFICIENT TUNG LEAVES

	Dry weight as percentage of original dry weight		N per 100 gm. dry leaves		Chlorophyll per 100 gm. dry leaves		N in dry fraction	
	Normal	Def.	Normal	Def.	Normal	Def.	Normal	Def.
	%	%	*gm.*	*gm.*	*gm.*	*gm.*	%	%
Original leaf	100.0	100.0	1.58	2.40	.349	.526	1.55	2.40
Extracted residue	35.5	34.0	0.46	0.57	.106	.142	1.13	1.66
Insol. at pH 9.0	11.6	2.6	0.11	0.09	.028	.021	0.86	3.49
Sol. at pH 9.0, insol. at pH 4.0	10.2	17.5	0.82	1.50	.125	.176	7.07	8.63
Sol. at pH 4.0	35.5	44.4	0.12	0.13	....	....	0.29	0.30
Total in fractions	92.8	98.5	1.51	2.29	.258	.339		
% Accounted for ..	92.8	98.5	95.1	95.3	74.0	64.5		

and normal leaves, hence it is concluded that copper-deficient tung leaves have a higher content of protein on a dry-weight basis than do normal leaves.

These proteins were subjected to amino acid assay by chemical and chromatographic technique. Chemical methods were generally unsatisfactory because of complicating substances present. A separation of the protein hydrolysates with phosphotungstic acid and silver salts gave somewhat variable results, with no outstanding differences evident in the content of basic nitrogen of the copper-deficient and the normal proteins. Filter-paper chromatography was used with the hydrolysate of isolated proteins and of acetone-extracted ground leaf. Both the descending method (2) and the more recent ascending method (13) were used.

The descending method was modified in some of the determinations by allowing the solvent to flow off the paper on a long run of three to four days, thus obtaining better separation of the slow-moving amino acids (fig. 3).

The faster-moving amino acids were lost in the process, so shorter runs were made for them. The following amino acids have been identified in the acid-hydrolyzed protein fractions and in hydrolysates from dried tung leaves by two-dimensional chromatography: aspartic acid, glutamic acid, serine, glycine, alanine, valine, the leucine group, phenylalanine, methionine, hydroxyproline, and proline. Also histidine and lysine were found colorimetrically (11). No evidence has been found for the presence of alpha-amino-

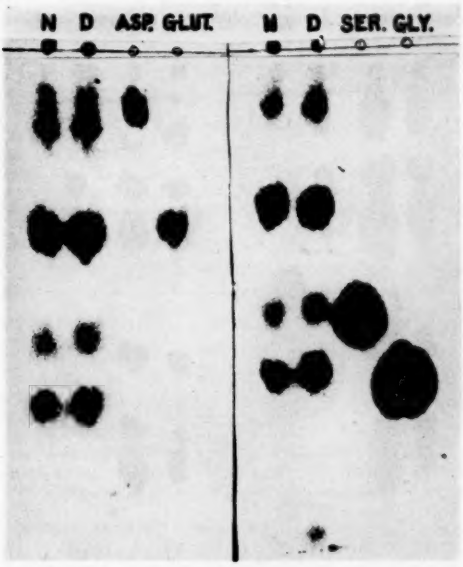


FIG. 3. Chromatograms of protein hydrolysates from normal (N) and from copper-deficient (D) tung leaves, showing identification of four amino acids (faster moving amino acids ran off paper).

butyric acid (fig. 4). The identified acids were present in approximately equal proportions in material from normal and from copper-deficient trees.

The conclusion drawn from extensive use of the chromatographic technique is that no significant difference can be detected in the quality of the principal proteins of copper-deficient and normal tung leaves, although a consistent difference was found in the quantity of protein. More recent work on intact chloroplasts isolated from similar material has been in agreement with the data on total protein.

The explanation of the quantitative difference in protein content found in normal and in copper-deficient plants may not reside in a direct role

played by copper in nitrogen metabolism, but rather in carbohydrate formation. It was previously shown for tung (**9**) that copper deficiency impairs apparent photosynthetic activity. Data from this laboratory are available which show that the copper of the leaves is mainly in the chloroplasts. This is in agreement with findings for other plants (**12**). Thus copper may be required in the enzymatic systems of photosynthesis. The impaired photosynthesis in copper deficiency along with unimpaired protein synthesis should result in the condition found in tung, namely, relatively high protein content and a low carbohydrate content.

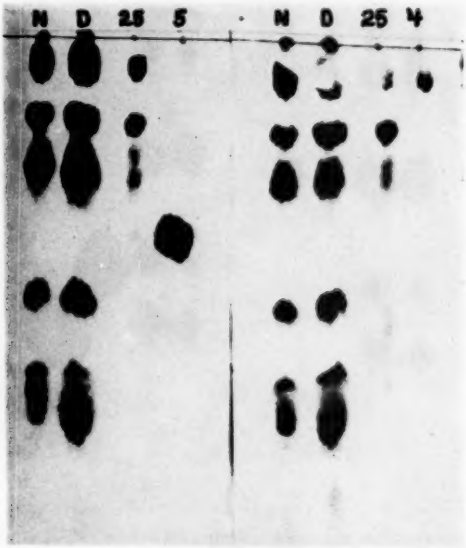


FIG. 4. Chromatograms of protein hydrolysate from normal (N) and from copper-deficient (D) tung leaves, also of a mixture of known amino acids (25) and of alpha-aminobutyric acid (5), showing the probable absence of alpha-aminobutyric acid in these protein hydrolysates.

The copper-nitrogen balance is not, therefore, due to an effect of copper on nitrogen metabolism. The converse, however, may be true: nitrogen may affect copper metabolism. Some data in support of the latter idea were obtained in a 2-level factorial experiment with N, P, and Cu on young tung trees, in which the roots were analyzed as well as the leaves. Only the main effects of N and Cu are presented in table II as P and the interactions were not significant. Confirming previous results, the data show that both N and Cu significantly influence copper deficiency. In this experiment N affected

the copper content of the leaves more than did Cu supply. In the roots, however, the copper content was significantly raised by Cu treatment but was not significantly affected by N treatment. These data are not extensive enough to be conclusive, but the indication is that the high-nitrogen plants did not translocate copper to the leaves as readily as did the low-nitrogen plants.

There is evidence in the literature that copper acts in the enzyme system in the ionic form. Ascorbic acid oxidase, for example, can be inactivated by dialysis of the copper and reactivated by copper ions (**8**). Copper ions applied in some fungicidal sprays appear to be inactivated by protein components of the spray (**7**).

TABLE II

THE EFFECTS OF NITROGEN AND COPPER SUPPLY ON COPPER-DEFICIENCY SYMPTOMS AND ON NITROGEN AND COPPER CONTENTS OF ROOTS AND LEAVES OF TUNG SEEDLINGS

Criterion*	Score per tree**	Leaves		Roots	
		Cu	N	Cu	N
	%	*p.p.m.*	%	*p.p.m.*	%
Mean of all treatments†	21.3	5.0	2.48	39.6	1.36
Cu, main effect	-10.0	1.9	-0.01	24.8	-0.02
N, main effect	31.0	-4.4	0.96	-5.3	0.30
L.D.Sig. at .05	6.6	3.2	0.18	9.6	0.07
L.D.Sig. at .01	9.0	5.1	0.29	15.1	0.11

* The factorial main effect is the average difference between all plants with and all plants without a given element. For example, the average copper content of the roots treated with copper was 24.8 p.p.m. higher than that of roots of plants that received no copper.

** Estimated percentage of the foliage showing symptoms of copper deficiency.

† Treatments in oz./tree were $CuSo_4 \cdot 5H_2O$, 2 oz. vs. 0, NH_4NO_3, 4 oz. vs. 1, superphosphate 8 oz. vs. 0. All trees received 1 oz. KCl, 1 oz. $ZnSO_4$. The treatments were applied to the soil on May 23 and July 10, 1945. Four replications were used of about 10 1-year-old seedlings per plot.

The effect of nitrogen on copper metabolism may thus result through the inactivation of copper ions by the increased protein content of the plants supplied with high nitrogen. The inactivation probably results from the formation of coordinate compounds with NH_2 groups characteristic of the amino acids and proteins. DAWSON and NAIR (**4**) have demonstrated such an inactivation of copper ions in plant material by such nitrogenous groups.

The inactivation of copper ions by proteins can be expressed by a dissociation constant,

$$K_1 = \frac{(\text{Cu ion})\ (\text{protein ion})}{(\text{Cu proteinate})},$$

for the reaction Cu ion + protein ion = Cu proteinate.

The concentration of the relatively stable copper proteinate will be relatively constant and the rate of reaction will depend primarily on the activities of the ionic species, or

$$K_2 = (\text{Cu ion})\ (\text{protein ion}).$$

This shows the copper activity to be inversely proportional to the protein ion concentration, which in turn is directly related to the nitrogen supply. Thus, on this hypothesis the copper–nitrogen balance is related to the inactivation of copper within the plant and to the impeded translocation from the roots to the leaves by the operation of mass-action principles.

Future work on copper deficiency should therefore give major consideration to two systems in the plant; the translocation of copper as affected by nitrogen concentration and the enzymatic relationships between copper and photosynthesis.

Summary

Further studies on the copper–nitrogen balance in tung are reported. A partial isolation of protein was made from leaves of normal and of copper-deficient tung trees. More protein was obtained per unit weight from copper-deficient leaves than from normal leaves. This confirms previous work in which nitrogen content was used as an index of protein content.

The proteins were analyzed for amino acid content by paper chromatography. Thirteen amino acids were identified in leaf proteins. No qualitative differences in amino acid content were found and no significant quantitative differences per unit weight of protein were observed, which indicates that the proteins isolated from normal and deficient leaves were not widely different chemically.

Preliminary data indicate that tung roots accumulate copper in direct relation to the copper supply and perhaps independently of the nitrogen supply. However, the nitrogen supply to the plant has an important effect in controlling the copper content of the leaves.

It is suggested that copper deficiency does not directly influence nitrogen metabolism or protein synthesis. The nitrogen supply to the plant, however, influences copper metabolism by inactivation of copper ions by protein ions. The mass-action principle is considered to be a probable basis of the copper–nitrogen balance required in fertilization of tung trees.

The author is indebted to Mr. Keat N. Thompson, scientific aid, for technical assistance and to Dr. E. J. Benne of Michigan State College for the calibration of the photelometer.

Division of Fruit and Vegetable Crops and Diseases
U. S. Department of Agriculture
Gainesville, Florida

LITERATURE CITED

1. Comar, C. L., Benne, E. J., and Buteyn, L. K. Calibration of a photoelectric colorimeter for the determination of chlorophyll. Ind. Eng. Chem., Anal. Ed. **15**: 524–526. 1943.
2. Consden, R., Gordon, A. H., and Martin, A. J. P. Qualitative analysis of proteins: A partition chromatographic method using paper. Biochem. Jour. **38**: 224–232. 1944.
3. Crook, E. M. The extraction of nitrogenous materials from green leaves. Biochem. Jour. **40**: 197–209. 1946.
4. Dawson, J. E. and Nair, C. K. N. Copper amalgam electrode. III. Cupric ion activities in plants. Abstr., 41st Meeting Amer. Soc. Agron. and Soil Sci. Soc. Amer., p. 68. 1949.
5. Gilbert, S. G., Sell, H. M., and Drosdoff, M. The effect of copper deficiency on the nitrogen metabolism and oil synthesis of the tung tree. Plant Physiol. **21**: 290–303. 1946.
6. Hamilton, J. and Gilbert, S. G. The relation of fertilization with copper and nitrogen to copper-deficiency symptoms, leaf composition and growth of tung. Proc. Amer. Soc. Hort. Sci. **50**: 119-124. 1947.
7. Heuberger, J. W. and Horsfall, J. G. Reduction in fungicidal value of copper compounds by organic materials. Phytopath. **32**: 370–378. 1942.
8. Kubowitz, F. Biochem. Zeit. **299**: 32. 1938. Cited in Elvehjem, C. A. and Wilson, P. W. Respiratory enzymes. P. 48. Burgess Publishing Co., Minneapolis, Minn. 1939.
9. Loustalot, A. J., Burrows, F., Gilbert, S. G., and Nason, A. Effect of copper and zinc deficiencies on the photosynthetic activity of the foliage of young tung trees. Plant Physiol. **20**: 283–288. 1945.
10. Lucas, R. E. Effect of copper on plant composition. Soil Sci. **65**: 461–469. 1948.
11. Macpherson, H. T. The basic amino acid content of proteins. Biochem. Jour. **40**: 470–481. 1946.
12. Neish, A. C. Studies on chloroplasts. II. Chemical composition. Biochem. Jour. **33**: 300–308. 1939.
13. Williams, R. J. and Kirby, Helen. Paper chromatography using capillary ascent. Science **107**: 481–483. 1948.

BRIEF PAPERS

THE FATE OF PHOSPHATASE, INVERTASE, AND PEROXIDASE IN AUTOLYZING LEAVES[1]

BERNARD AXELROD[2] AND ANDRÉ T. JAGENDORF[3]

Received December 11, 1950

When leaves are removed from a plant they continue to carry on many of their normal functions such as photosynthesis, carbohydrate and organic acid transformation, respiration, protein hydrolysis, etc. Yet, while these activities are being manifest there is a rapid and continuous decrease in the total leaf protein. Since the above reactions are mediated by enzymes which are themselves proteins, it becomes of interest to compare directly the survival of some enzymes in the autolyzing leaf with the survival of the total protein. Such information is of interest in connection with the general problems of leaf metabolism, but also in regard to the applied problems of storage behavior with certain agricultural products.

As a preliminary approach to the problem, a study of the comparative survival of several enzymes and of total soluble cytoplasmic protein was undertaken. It had previously been shown by WILDMAN and BONNER (**4**) that the soluble cytoplasmic protein of the leaves which they studied contained many enzymes. Tobacco was the plant of choice for the experiments described herein because of the detailed physicochemical data available on the cytoplasmic protein in its leaves (**5**). The present work is a part of a broader project carried out in this laboratory for the chemical and physical identification of the storage labile proteins of tobacco leaves (**3**).

The following enzymes were selected for study in the present work: phosphatase because of our special interest in its metabolic function, invertase and peroxidase because of the ease with which they could be determined.

Materials and methods

PLANT MATERIAL AND PROTEIN PREPARATION.—Nearly mature, well expanded leaves of *Nicotiana tabacum* var. Turkish Samsun were detached from the stem, randomized and stored under moist conditions at 25° C in darkness to accelerate protein disappearance. Groups of 50 to 60 leaves were withdrawn at 0, 1, 2, 4 and 7 days after storage. The leaves were then

[1] Enzyme Research Division Contribution No. 133.

[2] Biochemist, Enzyme Research Division, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture, Albany, California.

[3] Present address is Osborn Botanical Laboratory, Yale University, New Haven, Connecticut.

processed for whole soluble cytoplasmic protein according to the method of WILDMAN *et al.* (**5**); protein and total nitrogen was determined by the procedures of the same workers.

PHOSPHATASE ASSAY.—The procedure of AXELROD (**1**) was employed except that double the concentration of disodium nitrophenyl phosphate was employed to increase the range of linearity between extent of hydrolysis and quantity of enzyme. The units shown are arbitrary and are based on the optical density obtained in a Coleman spectrophotometer at 400 mμ using a 1.00 cm. square cuvette. Aliquots of reaction mixture were diluted 1 to 11 with alkali before reading.

INVERTASE ASSAY.—Invertase was determined by adding 5 ml. of an appropriate dilution of enzyme to 5 ml. of 10% sucrose in M acetate buffer, pH 5.0 and incubating at 37.5° C. Reducing sugar was determined by the method of HASSID (**2**). Results are expressed as ml. of .01 M ceric sulphate required by a 2-ml. aliquot of the reaction mixture after 30 minutes. In all cases 5 ml. of a 1 to 22 dilution of the original cytoplasm preparation was employed.

PEROXIDASE ASSAY.—Peroxidase was determined by noting the time required for a decrease in optical transmission of a standard reaction mixture from 100% to 75% in a 1.00 cm. square cuvette at 440 mμ. The reaction mixture consisted of 7.0 ml. H_2O, 0.5 ml. of 1 M acetate buffer pH = 5, 1.0 ml. 1% (v/v) aqueous guaiacol, 1.0 ml. 0.97% H_2O_2 (w/v) and 1 ml. of the necessary dilution of enzyme. The enzyme concentration was always so chosen as to give a reaction time whose reciprocal was in the linear range. The reaction was carried out at 25° C. Units are arbitrary, and are equal to the reciprocal seconds divided by ml. of preparation used in the test.

Results

From each batch of stored leaves, a preparation of the soluble cytoplasm was obtained by the procedure mentioned above. These preparations were analyzed for protein, total nitrogen, phosphatase, invertase and peroxidase. The data in table I show a decrease of approximately 45% in the amount of soluble cytoplasmic protein during the seven days of storage, but show little change in the total nitrogen. Despite the fact that the total protein has undergone considerable hydrolysis, it may be seen from table I that the content of the three enzymes studied has scarcely changed in this period. It would appear that the autolytic loss of protein which occurs in the cytoplasm of leaves during storage does not take place at the same rate for all of the constituent proteins, and that, in the case of the three enzymes studied, survival was essentially complete.

It is of course conceivable that the sustained level of the enzymes studied in the present experiment was due to a compensating increase in solubility during storage. The following experiment was designed to see if there was sufficient insoluble enzyme initially present to allow this expla-

TABLE I

FATE OF CYTOPLASMIC PROTEIN AND ENZYMES IN EXCISED TOBACCO LEAVES STORED AT 25° C IN THE DARK

Storage time days	Protein/ml.* mg.	Total N/ml. mg.	Phosphatase		Invertase		Peroxidase	
			Units/ml.	Units/mg. prot.	Units/ml.	Units/mg. prot.	Units/ml.	Units/mg. prot.
0	7.3	1.4	10.9	1.49	1.03	.14	0.98	.13
1	6.9	1.3	11.7	1.69	1.05	.15	1.12	.16
2	6.2	1.3	12.1	1.95	1.01	.16	1.19	.19
4	5.2	1.4	10.8	2.08	.89	.17	1.05	.20
7	4.0	1.5	10.1	2.52	.98	.25	1.24	.31

*In all cases 1 ml. of cytoplasm preparation represents approximately 0.36 leaves.

nation. Fresh tobacco leaves were ground in a colloid mill. A portion of the suspension was then freed of particulate matter in a basket centrifuge followed by centrifuging at 20,000 × g for 30 minutes. The activities of the suspension and the centrifugate are compared in table II. It is seen that there is no insoluble enzyme with the activity of phosphatase and invertase, and in the case of peroxidase the amount of non-extractable activity is so low that the above conclusion is not affected.

TABLE II

ENZYME ACTIVITIES OF SOLUBLE PROTEIN OF LEAF BREI COMPARED WITH WHOLE LEAF SUSPENSION

Enzyme	Relative activity in supernatant (Suspension = 100)
Phosphatase	96
Invertase	97
Peroxidase	81

The cytoplasm preparation from the 7-day leaves contained the three enzymes in a state of higher specific activity when expressed as units of activity per mg. protein than the preparation which had been made from unstored leaves. This suggests that the commonly accepted precaution of employing only fresh tissue for an enzyme isolation is not always advisable.

Conclusion

The loss of protein nitrogen in the cytoplasm of stored tobacco leaves is not reflected in a marked change in the contents of phosphatase, invertase and peroxidase even when the loss of protein amounts to 45% of that originally present.

It is a pleasure to acknowledge the assistance of Dr. Samuel G. Wildman and Miss Rosamond S. Baker.

KERCKHOFF LABORATORIES OF BIOLOGY
CALIFORNIA INSTITUTE OF TECHNOLOGY
PASADENA, CALIFORNIA

LITERATURE CITED

1. AXELROD, B. Citrus fruit phosphatase. Jour. Biol. Chem. **167**: 57–72. 1947.
2. HASSID, W. Z. Determination of sugars in plants by oxidation with ferricyanide and ceric sulfate titration. Ind. Eng. Chem., Anal. Ed. **9**: 228–229. 1937.

3. JAGENDORF, A. T. and WILDMAN, S. G. (Unpublished results.)
4. WILDMAN, S. G. and BONNER, J. The proteins of green leaves. I. Isolation, enzymatic properties and auxin content of spinach cytoplasmic proteins. Arch. Biochem. **14**: 381–413. 1947.
5. WILDMAN, S. G., CHEO, C. C., and BONNER, J. The proteins of green leaves. III. Evidence of the formation of tobacco mosaic virus protein at the expense of a main protein component in tobacco leaf cytoplasm. Jour. Biol. Chem. **180**: 985–1001. 1949.

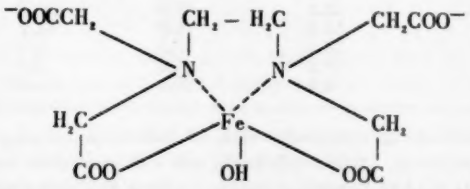
MAINTENANCE OF IRON SUPPLY IN NUTRIENT SOLUTIONS BY A SINGLE ADDITION OF FERRIC POTASSIUM ETHYLENEDIAMINE TETRA-ACETATE

LOUIS JACOBSON

Received October 30, 1950

When plants are grown in nutrient solution, invariably special attention must be paid to the problem of providing an adequate supply of available iron. Since iron is readily precipitated by high pH or phosphate, it is customary to add either organic or inorganic salts of iron to the nutrient solution at frequent intervals. HORNER, BURK, and HOOVER (**1**) developed a synthetic humate capable of holding iron in solution at moderately high pH and phosphate concentrations. Unfortunately, the humate is difficult to prepare and of indeterminate composition. EDGERTON (**2**) showed that by substituting metaphosphate for the usual orthophosphate in the nutrient solution, the resulting iron metaphosphate complex did not precipitate and was a satisfactory source of iron for plants. However, it may not always be desirable to use the meta instead of the orthophosphate and metaphosphoric acid as generally available contains large amounts of sodium.

Recently ethylenediamine tetra-acetic acid has become commercially available for use as a sequestrant. This substance forms chelated complexes especially with di- and tri-valent ions. SCHWARZENBACH and BIEDERMANN (**3**) represent the iron complex as:



This complex was examined as a source of iron for plants. Stock solutions were prepared by dissolving the free acid (Sequestrene AA—Alrose Chemical Co., Providence, Rhode Island) in KOH solution in the ratio of one molecule of acid (mol. wt. = 292 gm.) to two molecules of KOH. Iron, as ferrous sulphate, was added in the appropriate amounts to give varying molar ratios of iron to the complexing agent. The solution became quite acid on addition of the iron salt and was adjusted to pH 5.5 with KOH. In the presence of the complexing agent, ferrous iron is oxidized to the ferric form by atmospheric oxygen in a short time. In some cases, the complex was prepared by adding equi-molar ratios of the free acid and KOH to a suspension of freshly prepared excess $Fe(OH)_3$ in water. This was shaken

for several days until, after filtration, iron and nitrogen analyses indicated an equi-molar ratio of iron to the ethylenediamine tetra-acetate. The pH was 5.5.

The stability of the complex was tested by adding 5 p.p.m. iron, as the complex, to nutrient Hoagland solution at various pH values. The solutions were allowed to stand three months (no plants present) in the dark, and then the amount of iron left in the solution determined. No loss of iron occurred below pH 6. At pH 7, 18% of the iron had precipitated and at pH 8, 30% had come down. Even at pH 9, precipitation was not complete, about 10% remaining in the solution.

Tomato, sunflower, corn, and barley plants were grown in Hoagland solution (15 liters per five plants) in which the molar ratio of iron to the ethylenediamine tetra-acetate was varied, maintaining the iron constant at 3 p.p.m. The iron complex was added a day or two after the plants were transferred to the nutrient solutions and this was the only addition of iron throughout the experiment. The pH was not controlled and rose from an initial value of 5.5 to a final value of 7.5 or 8. The growth period was approximately six to eight weeks. The yield data are given in table I.

TABLE I

EFFECT OF RATIO OF IRON TO ETHYLENEDIAMINE TETRA-ACETATE ON THE GROWTH OF PLANTS. IRON CONTENT KEPT CONSTANT AT 3 P.P.M.

Molar ratio of iron to complex	Dry weight of plants (gm.)			
	Tomato	Sunflower	Corn	Barley
1:0.1	61.8	59.8	55.9	50.5
1:0.5	58.5	75.9	62.6	55.2
1:1	52.8	75.8	64.2	62.8
1:1.5	52.2	77.5	1.7	33.5
1:2	57.3	78.0	1.9	27.1
1:4	8.6	39.0	1.2	4.1

For general use, an equi-molar ratio of iron to complexing agent seems the most satisfactory. Below this ratio, iron will precipitate out of solution and the plants tend to become chlorotic. Above this ratio, such plants as corn and barley do not grow well.

In another series of experiments, the level of iron was varied, but the ratio of iron to complexing agent was maintained constant at equi-molar proportions. The experiment was carried out as before with plants in 15 liters of Hoagland solution and only one addition of iron. The growth period was approximately five weeks. The yield data are given in table II.

There was some chlorosis in the corn and barley plants grown in solutions containing less than 5 p.p.m. iron. Tomato and sunflower displayed chlorosis only at levels less than 1 p.p.m. Tomato and corn were also grown in solutions which contained from 5 to 100 p.p.m. iron in the form of ferric ethylenediamine tetra-acetate. At 100 p.p.m. iron, both plants were injured,

TABLE II

EFFECT OF CONCENTRATION OF IRON ETHYLENEDIAMINE TETRA-ACETATE ON GROWTH OF PLANTS. IRON AND COMPLEXING AGENT PRESENT IN EQUI-MOLAR PROPORTIONS.

P.p.m. iron as the complex	Dry weight of plants (gm.)			
	Tomato	Sunflower	Corn	Barley
0.01	11.1	28.2	11.3	4.9
0.10	69.6	74.2	26.8	18.4
1.0	76.7	74.5	64.1	24.1
3.0	74.5	77.1	64.0	23.5
5.0	77.7	77.8	62.3	30.6
10.0	82.7	76.2	63.0	33.3

especially in the early growth stages. At 50 p.p.m., some injury was apparent; at 25 p.p.m. or less, the plants grew well and were deep green.

It therefore appears that perhaps 5 or 10 p.p.m. iron as the ethylenediamine tetra-acetate complex provides adequate iron to the plant, is nontoxic and need be added only once.

It is convenient to prepare the complex by dissolving 26.1 gm. ethylenediamine tetra-acetic acid in 268 ml. of 1.0 *N* KOH, then adding 24.9 gm. $FeSO_4 \cdot 7\,H_2O$ and diluting to one liter. After aerating overnight to produce the stable ferric complex, the pH should be about 5.5. One ml. of this solution provides 5 p.p.m. to one liter of nutrient solution.

DIVISION OF PLANT NUTRITION
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA

LITERATURE CITED

1. HORNER, C. KENNETH, BURK, DEAN, and HOOVER, SAM R. Preparation of humate iron and other humate metals. Plant Physiol. **9**: 663–670. 1934.
2. EDGERTON, L. J. The use of metaphosphate in nutrient solutions. Proc. Amer. Soc. Hort. Sci. **41**: 237–240. 1942.
3. SCHWARZENBACH, G. and BIEDERMANN. Komplexone IX. Titration von metallen mit athylenediamin-tetraessigsaure H_4Y. Endpunktsindikation durch ph-Effekte. Helv. Chim. Acta. **31**: 459–466. 1948.

DISTRIBUTION OF MICRONUTRIENT METALS IN LEAVES AND CHLOROPLAST FRAGMENTS[1]

F. R. Whatley, Lawrence Ordin and Daniel I. Arnon

Received January 4, 1951

An investigation of the extracellular reactions of chloroplasts in the light (**4**) and in the dark (**5**) has focused attention on the possible catalytic role of one or more of the micronutrient metals: iron, manganese, copper, zinc, and molybdenum in the cellular activities of these cytoplasmic bodies. The consideration of a possible participation of one or more of the micronutrient metals involves the knowledge of their relative distribution in the leaf and chloroplasts. This communication reports results of analyses undertaken with this purpose in view.

Methods

Sugar beet and chard plants were grown in a greenhouse in aerated nutrient solutions of the composition previously described (**2**), supplemented with the micronutrients B, Mn, Cu, Zn and Mo (**1**). Large leaves from plants approximately 12 weeks old were used for chemical analysis and the isolation of chloroplast fragments. The chloroplast fragments were prepared by trituration of the leaves in the cold without dilution in a "Vitajuicer" (**3**) and subsequent differential centrifugation as previously described (**2**), except that the final centrifugation was carried out in a Sorvall Type SS–1 centrifuge, for two hours in the cold, at the maximum speed corresponding to approximately 20,000 g. (A special test disclosed that the Vitajuicer could contribute only insignificant amounts of metal contamination to the analysis.) The chloroplast fragments were dried in an all-glass lyophilizer, and the dried material was kept in the cold in screw-capped bottles. The analyses of the whole leaves were made on samples of fresh leaves which were heated in the autoclave at 5 lbs. pressure for five minutes to stop enzymatic activity and were subsequently dried at 70° C. The dry material was ground in a porcelain mortar and redried at 70° C prior to analysis. The analytical determinations were made on aliquots of the ground leaves and of the lyophilized chloroplast fragments.

Chlorophyll was measured as previously described (**2**). The total nitrogen, found by the micro-Kjeldahl method, in the trichloracetic acid precipitate of a water suspension of the dried material was designated as protein nitrogen and was multiplied by the factor 6.25 to give protein values. The metals were determined by the procedures of Parks *et al.* (**15**) modified as follows: in the preparation of the "ash solution" the hydrofluoric acid step was omitted and sulphuric acid was used in the perchloric acid digestion;

[1] This work was supported in part by a grant from the United States Public Health Service.

molybdenum was determined by the thiocyanate-stannous chloride method as described by SANDELL (17) except that isoamyl alcohol was used as the extracting solvent; in the iron procedure sodium acetate buffer (pH 3.5) was added to facilitate the adjustment of pH with ammonium hydroxide using Congo red as indicator; and for copper, carbon tetrachloride rather than isoamyl acetate was used for the final extraction of the diethyldithiocarbamate complex.

Results and discussion

The previously published data on the composition of chloroplasts have been thoroughly reviewed by RABINOWITCH (16). In the present investigation computations made from the chlorophyll content of leaves show that the chloroplasts represent over a third of the dry weight of leaves (table I),

TABLE I

CHLOROPHYLL-PROTEIN RATIO (Chl : P) IN CHLOROPLAST FRAGMENTS (chl.fr.).

	Dry wt. basis	
	Sugar beets	Chard
% Chlorophyll	5.15	5.41
% Protein	40.7	40.9
Chl : P, mass ratio*	0.127	0.132
Chl : P, molecular ratio**	2.38	2.48
Chloroplasts as % of dry leaves†	35.4	37.4

*Grams chlorophyll to grams protein.

**Moles chlorophyll per Svedberg unit of protein (M.W. 17,000).

†Dry sugar beet leaves and chard contained 1.82 and 2.02% chlorophyll respectively. The percentage of the total leaf dry weight represented by chloroplasts was computed from the formula $\frac{\%\text{ chlorophyll in leaves.}}{\%\text{ chlorophyll in chl. fr.}} \times 100$.

a value in agreement with that of NEISH (14). The values of somewhat over 5% for chlorophyll in chloroplasts reported in table I agree with those reported by BOT (6) and COMAR (8) but are lower than those of MENKE (13), SMITH (18), GRANICK (9) and WARBURG (19, p. 205). The protein content (over 40%) of chloroplasts is within the range reported by BOT (6), CHIBNALL (7), MENKE (12), NEISH (14) and COMAR (8). The chlorophyll : protein ratio is of considerable theoretical interest in that it bears on the existence of a chlorophyll-protein complex in the chloroplasts. The "molecular" chlorophyll : protein ratios based on a Svedberg protein unit of 17,000, reported in table I, fall within the range of most of the values computed for chloroplasts by Rabinowitch from the data of other investigators (16, p. 390). Further support is thus offered for the view that there is not enough protein in the chloroplasts to provide each chlorophyll molecule with its own (Svedberg) unit of protein in a manner analogous to cytochrome C, for example, in which one molecule of hematin is linked with one unit of protein.

The results of metal analyses for sugar beets are given in table II. The data for chard, omitted here for the sake of brevity, were in substantial agreement. In comparing the data of different investigators, recognition must be given not only to differences in species, varieties, and analytical methods, but to variability among factors of the climatic and nutrient environment which influence the inorganic composition of plants. The results in table II are reported not only in terms of concentration but also in terms of a molecular ratio between chlorophyll and metal. The data indicate that of the five metal micronutrients in the leaf only iron and copper are concentrated in the chloroplasts. These findings are in general agreement with those of NEISH (**14**) who found these two metals concentrated in the chloroplasts and with those of HILL and LEHMANN (**10**) who found most of the leaf iron to be present in the chloroplasts. The data of NEISH (**14**) provide the only previous account known to us of copper distribution in chloroplasts.

TABLE II

DISTRIBUTION OF MICRONUTRIENT METALS IN LEAVES AND CHLOROPLAST FRAGMENTS OF SUGAR BEETS

Dry wt. basis

	Leaves		Chloroplast fragments		% of total metal in leaf
	Micrograms / Gram	Moles chlorophyll / Gr. atom metal	Micrograms / Gram	Moles chlorophyll / Gr. atom metal	
Iron	132.5	8.5	230.	13.8	61.4
Copper	14.7	86.7	26.5	136.5	63.8
Manganese	225.	4.9	226.	13.8	35.5
Zinc	65.3	20.1	43.5	85.6	23.5
Molybdenum	1.5	1290.	1.04	5260.	24.5

The manganese, zinc, and molybdenum were found not to be concentrated in the chloroplasts. The concentration of manganese in the chloroplasts was approximately equal to, but that of zinc and molybdenum was less than that expected on the basis of random distribution in the leaf. The values for manganese and zinc content of chloroplast fragments are in general agreement with those obtained by WARBURG (**9**) for chloroplast "granules." We know of no previous account of molybdenum distribution in chloroplasts.

The relatively high concentration of iron and copper suggests that these metals play a special role in the enzymatic activities of chloroplasts. Nevertheless, composition data, however suggestive, cannot by themselves provide proof for the participation of a micronutrient element in a cellular reaction. Neither is it considered impossible, despite their relatively low concentrations, for either manganese, zinc, or molybdenum to serve as catalysts in some chloroplastic activity. Conclusions of this nature must be substantiated by other lines of evidence. This subject is treated in greater detail elsewhere (**4**).

Summary

The iron, copper, manganese, zinc, molybdenum, and chlorophyll content of leaves and isolated chloroplast fragments of sugar beets was determined. Of the five metal micronutrients, iron and copper were found to be concentrated in the chloroplasts.

We wish to thank Dr. C. M. Johnson for his helpful suggestions regarding analytical procedures.

DIVISION OF PLANT NUTRITION
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA

LITERATURE CITED

1. ARNON, D. I. Microelements in culture-solution experiments with higher plants. Amer. Jour. Bot. **25**: 322–325. 1938.
2. ARNON, DANIEL I. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. **24**: 1–15. 1949.
3. ARNON, DANIEL I. and WHATLEY, F. R. Factors influencing oxygen production by illuminated chloroplast fragments. Archiv. Biochem. **23**: 141–156. 1949.
4. ARNON, DANIEL I., WHATLEY, F. R., and CARLTON, ALAN H. The participation of metals in the light reactions of photosynthesis. (In preparation.) 1951.
5. ARNON, DANIEL I., WHATLEY, F. R., and MAZELIS, MENDEL. Oxygen uptake and carbon dioxide evolution in the dark by chloroplast fragments and leaf extracts. (In preparation.) 1951.
6. BOT, G. M. The chemical composition of chloroplast granules (grana) in relation to their structure. Chronica Botanica **7**: 66–67. 1942.
7. CHIBNALL, A. C. Protein metabolism in the plant. Yale University Press, New Haven. 1939.
8. COMAR, C. L. Chloroplast substance of spinach leaves. Bot. Gaz. **104**: 122–127. 1942.
9. GRANICK, S. Chloroplast nitrogen of some higher plants. Amer. Jour. Bot. **25**: 561–567. 1938.
10. HILL, R. and LEHMAN, H. Studies on iron in plants with special observations on the chlorophyll : iron ratio. Biochem. Jour. **35**: 1190–1199. 1941.
11. MENKE, W. Untersuchung der einzelnen Zellorgane in Spinatblättern auf Grund praparativchemischer Methodik. Zeit. für Bot. **32**: 273–295. 1938.
12. MENKE, W. Untersuchungen über das Protoplasma grüner Pflanzenzellen. I. Isolierung von Chloroplasten aus Spinatblättern. Zeit. Physiol. Chem. **257**: 43–48. 1938.

13. MENKE, W. Untersuchungen über das Protoplasma grüner Pflanzenzellen. II. Der Chlorophyll-gehalt der Chloroplasten aus Spinatblättern. Zeit. Physiol. Chem. **263**: 100–103. 1940.
14. NEISH, A. C. Studies on chloroplasts. II. Their chemical composition and the distribution of certain metabolites between the chloroplasts and the remainder of the leaf. Biochem. Jour. **33**: 300–308. 1939.
15. PARKS, R. Q., HOOD, S. L., HURWITZ, C., and ELLIS, G. H. Quantitative chemical microdetermination of twelve elements in plant tissue. Ind. Eng. Chem., Anal. Ed. **15**: 527–533. 1943.
16. RABINOWITCH, EUGENE I. Photosynthesis and related processes. Interscience Publishers, New York. 1945.
17. SANDELL, E. B. Colorimetric determination of traces of metals. Interscience Publishers, N. Y. 1944.
18. SMITH, E. L. The chlorophyll-protein compound of the green leaf. Jour. Gen. Physiol. **24**: 565–583. 1941.
19. WARBURG, O. Heavy metal prosthetic groups and enzyme action. Oxford Press. 1949.

NOTES

Charles Reid Barnes Honorary Life Member.—The 1950 Barnes Honorary Life Membership of the A. S. P. P. was conferred upon Dr. John W. Shive in recognition of his outstanding record as an investigator and many years of service in the realm of plant physiology. It is a great privilege for the Society to honor an already distinguished member. In 1938 Dr. Shive received the Stephen Hales Award and during 1939–40 he served as president of the A. S. P. P. Despite his emeritus status he continues as an active teacher and investigator. John Wesley Shive was born in Halifax, Pennsylvania, on February 13, 1877. He attended Dickinson College, receiving a bachelorate in philosophy in 1906 and master of arts in 1907. He was a research fellow at Johns Hopkins from 1913 to 1915, receiving a doctorate in philosophy in 1915. He was honored with a doctorate in science from Rutgers University in 1946. From 1906 to 1911 he was an instructor at Perkiomen Boys' School. In 1915 he was appointed Plant Physiologist in the Experiment Station at Rutgers; in 1921 he became an Associate Professor of Plant Physiology at the University, and served as Professor from 1923 to 1946. Since 1946, he has been Emeritus Professor. In addition to his academic and research activities at Rutgers, he served as an assistant in the laboratory of Plant Physiology of the Carnegie Institution in 1913 and as a collaborator of the U. S. Regional Plant, Soil, and Nutritional Laboratory from 1940 to 1946. He is a member of the American Association for the Advancement of Science, the Botanical Society of America, the Society of Experimental Biologists, the American Society of Plant Physiologists, and the American Institute of Biological Sciences. His major interest and contribution in plant physiology has been in the salt requirements of agricultural plants and other phases of plant nutrition.

Stephen Hales Award.—The American Society of Plant Physiologists awarded the Stephen Hales prize for 1950 to Dr. Birgit Vennesland for her excellent studies on the pathways of carbon dioxide release during respiration. Dr. Vennesland has made outstanding contributions to our knowledge of the nature of the enzymes involved in the respiration of plant tissues and she has shown that the same enzymes can also catalyze the dark fixation of carbon dioxide.

Western Section Meeting.—The Western Section of the American Society of Plant Physiologists will meet with other affiliates of the Pacific Division, A.A.A.S., at the University of Southern California, Los Angeles, June 19–22,

1951. The program will consist of four sessions of submitted papers, a field trip, and two symposia: "Air Pollution and Plant Life" and "Physiological Genetics of Higher Plants." Members of the Society who may be able to attend should correspond at once with the Secretary and Program Chairman of the Western Section to receive further notices or to submit papers for presentation. Address: Professor Harlan K. Pratt, Division of Truck Crops, University of California, Davis, California.

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS

Organized in 1924 for the advancement of Plant Physiology as a pure and applied field of botanical science

---

OFFICERS FOR 1950–51

President: Dr. Kenneth V. Thimann,
Harvard University, Cambridge, Massachusetts

Vice-President: Dr. Daniel I. Arnon,
University of California, Berkeley, California

Secretary: Dr. T. C. Broyer,
University of California, Berkeley, California

Executive Secretary-Treasurer: Dr. J. Fredrik Stanfield,
Miami University, Oxford, Ohio

Any one actively interested in Plant Physiology is eligible for membership in the Society. Candidates for membership must be recommended by two members, and approved by executive committee, or by the vote of the Society at some annual or special meeting.

The payment of the annual dues to the Society entitles the members to receive the official journal, Plant Physiology. They will receive in addition such circular bulletins and announcements sent out periodically by the secretary. Members are invited to take a personal interest in the growth and development of the Society.

Manuscripts for Plant Physiology should be addressed to Prof. Warren H. Loomis, Department of Botany, State University of Iowa, Iowa City, Iowa. Drawings and photographs should not exceed 8.5 × 11 inches in size, should be numbered correctly on the back, and separate typewritten legends provided for the text of the manuscript. Tables and cuts should be used sparingly and the text presented in concise form. Instructions to authors for the preparation of manuscripts are given in the January number, Volume 25, of Plant Physiology, pages 205–208.

CONTENTS—(Continued)

Photoelectric-Type Integrating Light Recorders: A Summary of Performance Over a Five-Year Period. G. F. Sprague and K. C. [illegible], U. S. Plant, Soil, and Nutrition Laboratory, Ithaca, New York ..... [illegible]

Bound Water in Aspergillus niger. G. W. Tims and J. [illegible], University of Missouri, Columbia, Missouri 324

The Sterols of Alfalfa and Certain Cereal Grasses. R. E. [illegible], L. D. Markham, and R. E. Buxton, Kansas Agricultural Experiment Station, Manhattan, Kansas 327

Some Effects of Decapitation on Electrical and Elongation Phenomena in the Avena Coleoptile. K. B. Mitra and A. R. Schmitz, University of Texas, Austin, Texas 333

Availability of Soil Moisture for Active Absorption in Alfalfa. T. J. Army and T. T. Kozlowski, University of Massachusetts, Amherst, Massachusetts 338

Effects of Flooded Soil on Growth of Pine Seedlings. P. [illegible], Kentucky Division of Forestry, Stanford, Kentucky 345

The Relationship of Structure and Plant-Growth Activity in Substituted Benzoic and Phenoxyacetic Acids. R. M. Muir and [illegible] Hamner, State University of Iowa, Iowa City, Iowa, and [illegible] College, Claremont, California 350

Investigations on Nitrogen Compounds and Nitrogen Metabolism in Plants. I. The Reaction of Nitrogen Compounds with Ninhydrin on Paper; A Quantitative Procedure. J. F. Thompson, F. M. Eaton, and F. C. Steward, University of Rochester, Rochester, New York 355

A Biochemical Basis for Copper-Nitrogen Balance in Tung. A. S. [illegible], U. S. Division of Fruit and Vegetable Crops and Diseases, Gainesville, Florida 363

Brief Papers:

The Fate of Phosphatase, Invertase, and Peroxidase in Senescing Leaves. Bertram Axelrod and A. T. [illegible], California Institute of Technology, Pasadena, California 368

Maintenance of Iron Supply in Nutrient Solutions by a Single Application of Ferric Potassium Ethylenediamine Tetra-Acetate. Lloyd [illegible], University of California, Berkeley, California 411

Distribution of Micronutrient Metals in Leaves and Chloroplast Fragments. F. R. Whatley, Lawrence Odell, and D. I. [illegible], University of California, Berkeley, California 414

Notes 419